Emx1 null mutant mouse phenotype: potential implications for human epilepsy

Thesis

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Emx1 null mutant mouse phenotype: potential implications for human epilepsy

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ABBREVIATIONS

AEC	3-amino-9ethil carbazole (AEC).
Bp	base pair
BrdU	bromo-deoxyuridine
°C	degree Celsius
Ci	Curie
CNS	Central nervous System
Cpm	counts per minute
DAB	diaminobenzidine tetrahydrochloride
DNA	deoxyribose nucleic acid
kb	kilobase pair
M	Molar
μ	micro
dNTP	deoxynucleotide triphosphate
OD	optical density
^{32}P	phosphate isotope
RNA	ribonucleic acid
rpm	revolutions per minute
^{35}S	sulphur isotope
w/v	weight to volume

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To my beloved parents

ABSTRACT

In the last decade, several homeobox-containing genes including members of the empty spiracle (Emx1 and Emx2) and orthodenticle (Otx1 and Otx2) families, have been found to be expressed in the cerebral cortex of rodents. Among these genes, Emx1 shows the most widespread pattern of expression in this part of the brain. It has been recently demonstrated that Emx1 is expressed by pyramidal cells, which are the glutamate-containing projection neurons of the cortex, from the time of their origin during embryonic period of neocorticogenesis, and until long after birth.

In contrast, non-pyramidal cells, the GABA-containing cortical interneurons originate in the ventral telencephalon and have been found not to express this homeobox gene.

Emx1 gene inactivation at a first analysis has not showed to cause gross morphological abnormalities to the developing and the adult brain.

We studied the development of the telecephalon in Emx1 null embryos and examined the cortices of adult Emx1 null mice, using standard histological methods as well as a variety immonocytochemical, in situ hybridization and tracing techniques. I did not find gross abnormalities in the development of Emx1 null mutant embryos; therefore, the overall cytological appearance of the adult cortex was indistinguishable between the mutant mice and the wild type littermates.

A deeper analysis of the density and distribution of the two principal neuronal cell types of the cortex, pyramidal and nonpyramidal neurons, revealed no significant difference in the number and layering of pyramidal neurons in mutant cortices. However, counts of GABAergic neurons showed statistically significant differences between mutant and wild type littermates.

Earlier reports provided evidence in support of the hypothesis that loss of inhibitory GABAergic interneurons results in neuronal hyperxcitability in the cortex, and eventually leads to seizure activity; more recent investigation confirmed that disruption of GABAergic neurotransmission is implicated in epilepsy.

Consequently, the hypothesis that loss of GABAergic interneurons in Emx1 null mice would result in neuronal hyperexcitability leading to seizure activity, arose.

Baseline electroencephalographic recordings obtained from Emx1 mutant mice and normal littermates revealed showed epileptic-like activity characterized by abnormal spikes in all $Emx1^{-/-}$ animals.

To gain insight into the possible involvement of Emx1 gene in human epilepsy, patients affected by different epileptic syndromes were analysed. A point missense mutation in the second exon of Emx1 gene was found in all affected individuals of two different families. Those patients were affected by epilepsy with different phenotypes, that could be included into the wider group of idiophatic generilized epilepsy.

These results constitute the first evidence that a homeobox gene, supposed to have a direct role in orchestrating cortical development, may be involved in human epilepsy. Indeed, idiophatic epilepsies may be viewed as developmental disorders in which the selective inactivation of a homeogene leads to a reduction of inhibitory interneurons in the cerebral cortex, hence, to increased neuronal excitability.

The data coming from a knock-in mouse for this mutation could give information about the underlying molecular mechanisms.

Chapter 1

INTRODUCTION

1.1 DEVELOPMENT OF THE VERTEBRATE CENTRAL NERVOUS SYSTEM

The target of neuroscientists is to understand the molecular mechanisms underlying human motor, sensory, and cognitive abilities. To reach this target the first question to answer is "how the brain develops".

All behavioral tasks performed by the mature nervous system depend on precise interconnections of many millions of neurons. These connections are made during embryonic and postnatal development.

The development of the vertebrate nervous system can be subdivided into seven fundamental steps: induction of neural plate; regionalization of the neural tube along the dorsoventral and anterior-posterior axis; generation of neurons and glia from multipotential precursors; apoptotic cell death; migration of neurons; guidance of axons to their targets; formation of synapse. This developmental program culminates in great variety of neural cell types, both neurons and glial cells (reviewed in Jessell and Sanes, 2000).

There are three principal layers of cells in the mammalian embryo (figure 1.1): the *endoderm*, the innermost layer, gives rise to the gut, lungs and liver; the *mesoderm*, the middle layer, gives rise to the connective tissue, muscle, and the vascular system; and the *ectoderm*, the outermost layer, gives rise to the major tissues of the central and peripheral nervous system.

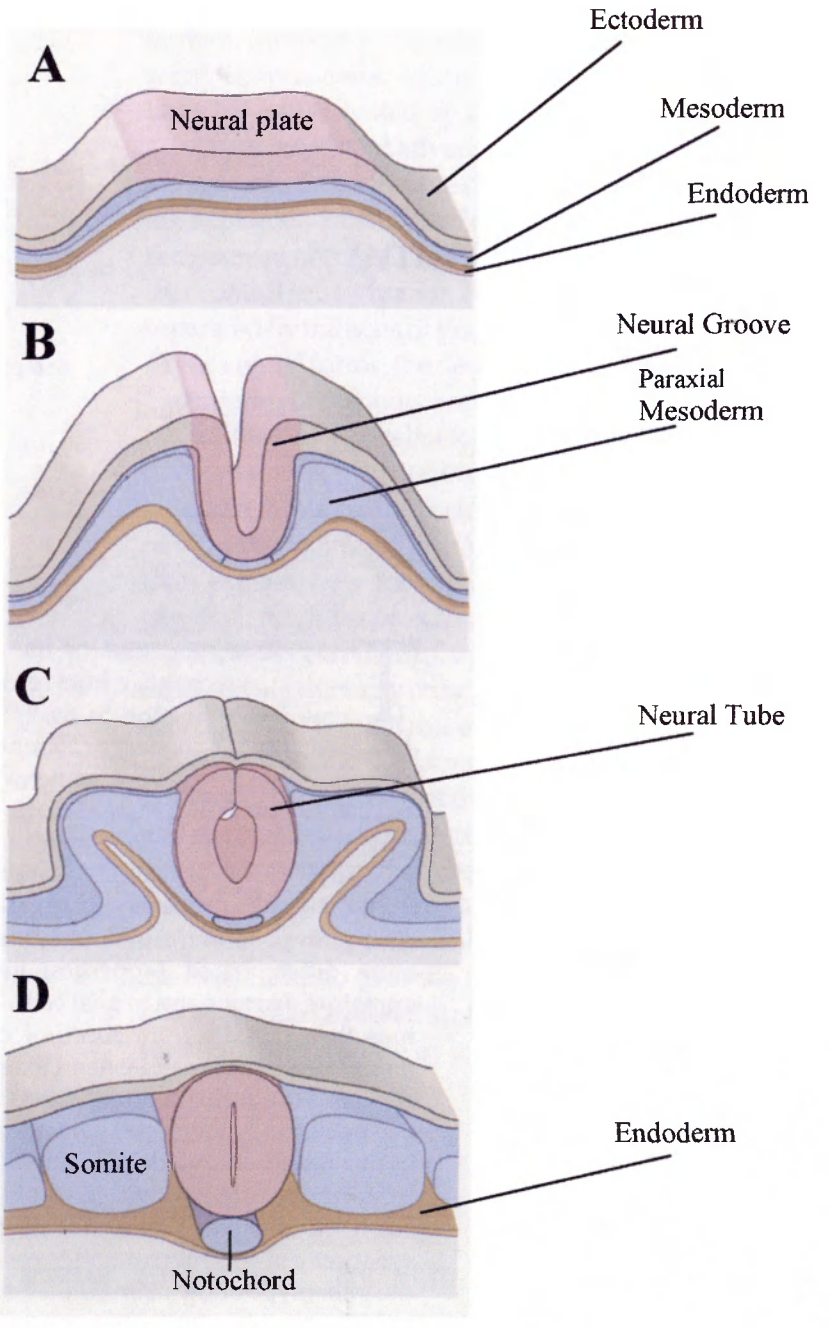


Figure 1.1
Diagram showing the neural plate that folds in stages to form the neural tube. A. Position of neural plate in relation to the ectoderm, mesoderm and endoderm. B. Folding of the neural plate to form the neural groove. C. Dorsal closure of the neural folds to form the neural tube. D. Maturation of the neural tube.

Neural and glial cells derive from a sheet of ectodermal cells located along the dorsal midline of the embryo at the gastrula stage. As this ectodermal sheet begins to acquire neural properties it forms the *neural plate* and its cells become distinguishable by their columnar appearance. Ectodermal cells that fail to follow the neural program of differentiation give rise to the epidermis and the skin .

Soon after the neural plate has formed, it begins to fold into a tubular structure (figure 1.1), the *neural tube,* through a process called neurulation. The neural tube is a hollow structure and its cavity gives rise to the ventricular system of the central nervous system. The caudal region of the neural tube will give rise to the spinal cord, and the rostral region becomes the brain. The epithelial cells that line the wall of the neural tube generate virtually all the neurons and the glial cells of the central nervous system, and are therefore called neuroepithelium. Only the nerve cells of the periphery, whose cell bodies lie outside the spinal cord originate from the neural crest, a population of cells originally located at the lateral margins of the neural plate. During these early stages of neural development cells divide rapidly. However, the extent of cell proliferation is not uniform along the length of the neural tube. Individual regions of the neural epithelium expand at different rates and begin to form the various specialized regions of the mature central nervous system.

Even before the posterior part of the neural tube has formed, the most anterior portion of the tube is undergoing to drastic changes. Indeed, the proliferation of cells in the rostral part of the neural tube initially forms three brain vesicles: the prosencephalon or forebrain, the mesencephalon or midbrain, the rombencephalon or hindbrain (figure 1.2). Later in development two structures emerge from the primitive forebrain: the telencephalon, which gives rise to the cerebral hemispheres, and the diencephalon, composed primarily of the thalamus and the hypothalamus. In this later stage of development the mesencephalon remains undivided. The rombencephalon gives rise to two structures: the metencephalon, consisting of the pons and the cerebellum, and the myelencephalon or medulla. The caudal portion of the neural tube remains undivided and becomes the spinal cord (figure 1.2). Thus, the five brain vesicles and the spinal cord define the six major divisions of the mature central nervous system.

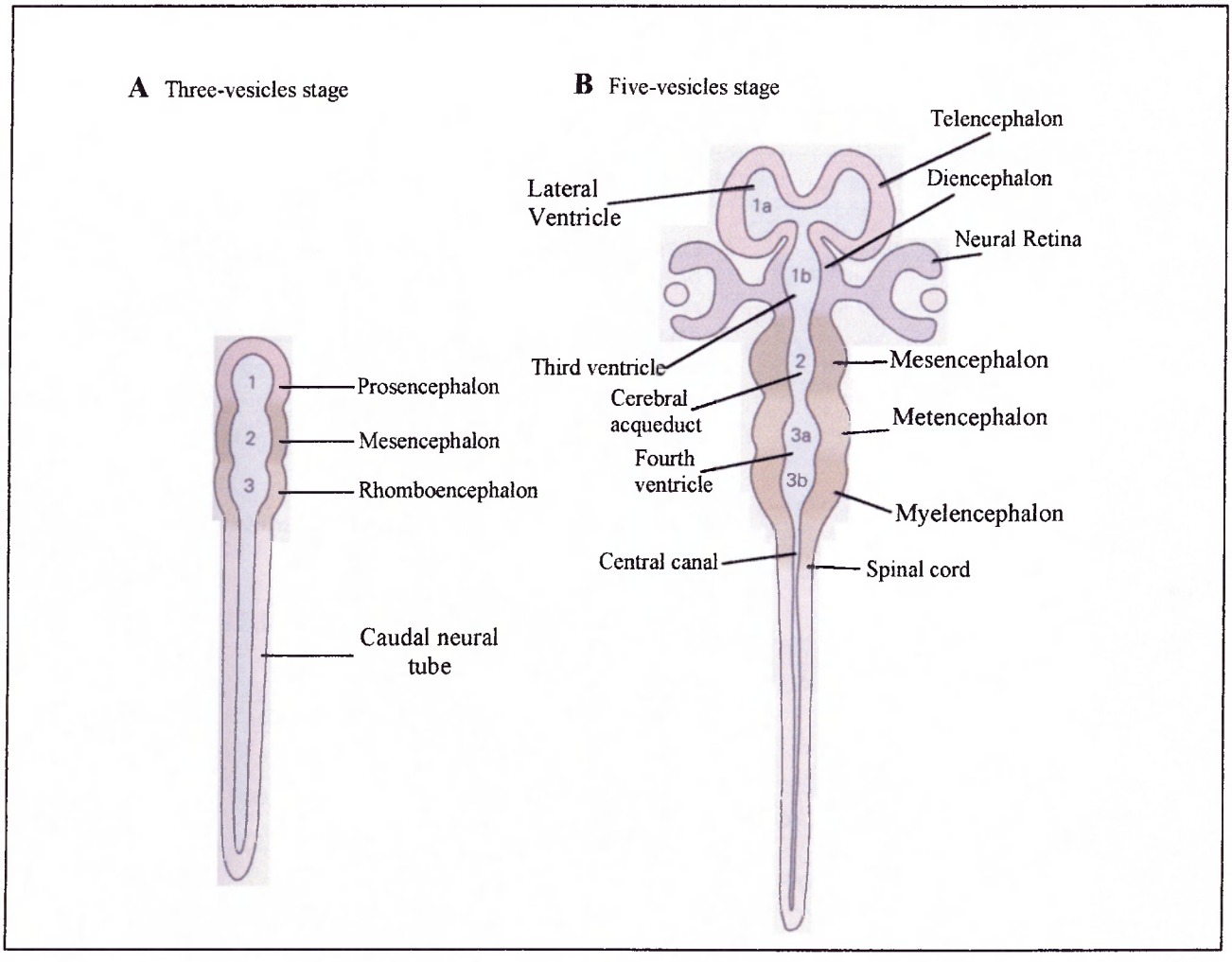


Figure 1.2

Successive stages in the development of the neural tube. A. At early stages of development only three vesicles are present. B. At later stages two additional vesicles form: the prosencephalon gives rise to telencephalon and diencephalon, whereas the rhomboencephalon to mesencephalon and metencephalon. Telencephalon and diencephalon constitute the so-called forebrain, the mesencephalon is also referred as midbrain, finally the mesencephalon and the metencephalon form the hindbrain.

1.1.1 Neural induction

The differentiation of cells in nervous system as in other organs is the consequence of a complex program that directs the expression of specific genes within individual cells. Two major groups of factors determine which genes are expressed in a cell. The first group is composed by the so-called *inducing factors*; those are signalling molecules provided by other cells. The second group of factors is formed by molecules that are activated or induced in cells upon the exposure to an inducing factor from another cell.

A fundamental step in neural development is the allocation of a group of ectodermal cells as precursors of the entire nervous system. This process involves an inductive interaction first appreciated by Spemann and Mangold 1920s: they made the fundamental discovery that the differentiation of the neural plate from uncommitted ectoderm in amphibian embryos depends on signals secreted by a specialized group of cells later called *organizer region.* For decades the identity of endogenous inducing factors remained obscure. However, during the early and mid-1990s studies in embryos of the frog Xenopus leavis have dramatically advanced our understanding of neural induction.

In absence of any extrinsic factor, ectodermal cells would acquire a neural fate by "default" (figure 1.3). However, the capacity of ectodermal cells to undergo neural differentiation is suppressed by signals transmitted between neighboring cells. The mediators of this suppressive signal are members of a family of secreted factors implicated in skeletal development, the bone morphogenetic proteins (BMPs). By contrast, the inductive signals from the organizer region can induce neural tissue by blocking BMP signalling. Indeed, the cells in the organizer region express three secreted proteins-follistatin, noggin, and chordin each of which is able to induce Xenopus ectoderm to differentiate into neural tissue. All three proteins appear to act by binding to BMPs and inhibiting their activity (figure 1.3).

Near the end of 1990s, studies on mouse mutants lacking genes encoding candidate neural inducers expressed by the organizer region (the node in mouse) have shown that there is still neural differentiation. Moreover, genetic elimination of the mouse node in its entirety similarly failed to block neural differentiation.

It now appears that neural induction begins prior to the formation of the organizer region and thus must be initiated by signals derived from other cell types. In addition, members of other families of signalling molecules, notably the fibroblast growth factors (FGFs) have now been proposed as early-acting factors that initiate neural induction. So the suppression of BMP signalling may maintain rather than initiate the process of neural differentiation.

FGFs have been shown to function, at least in part, by repressing the expression of BMP genes at a transcriptional level, in contrast to the organizer-derived factors such as noggin and chordin, which act extracellularly to block BMP activity.

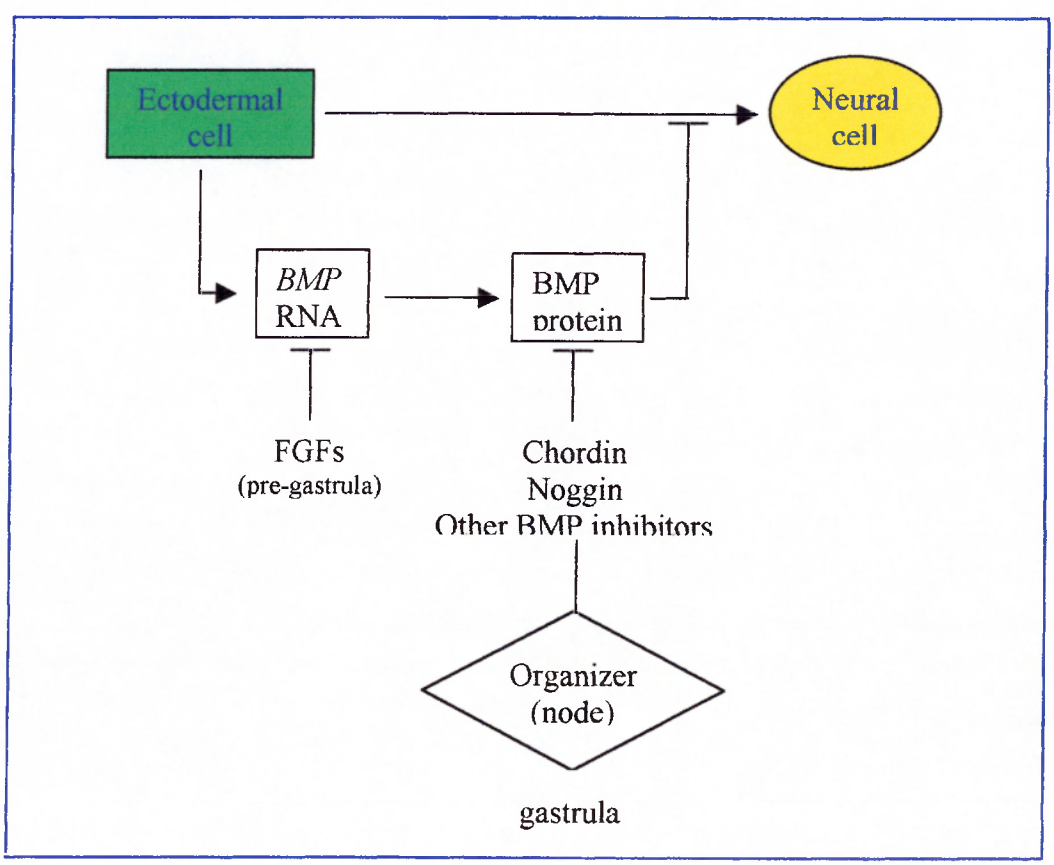


Figure 1.3
A molecular pathway for neuronal induction in the vertebrate embryo. (For details, see text.)

1.1.2 Regionalization of the neural tube

The cells in the neural tube acquire their regional identity by virtue of the position that they occupy along the two primary neural axes: dorsoventral and anteroposterior. In other words, the position of a cell defines its identity through the exposure to regionally restricted signalling factors that operate over these neural axes.

A relatively small number of signalling factor families - TGF-βs (including BMPs), hedgehogs, FGFs, Wnts and retinoids- can account for many features of regional cell specialization within the neural tube.

Along the dorsoventral axis, the two primary signalling factors appear to belong to the hedgehog and BMP families. Neurons of the mature spinal cord serve two major functions: they process sensory input and coordinate motor output. These two subsets of spinal cord neurons are segregated anatomically. Neurons involved in the processing of sensory input are located in the dorsal half of the spinal cord, whereas those involved in motor output are located in the ventral half.

These cell types are generated at different positions along the dorsoventral axis of the neural tube at early stage of spinal cord development. Cell differentiation in both dorsal and ventral halves of the neural tube is controlled by inductive signals. Ventral patterning is regulated by the activity of a single protein, SHH, which generates different cell types at different concentration. In contrast, dorsal patterning appears to involve several members of the BMP family, each of which may induce a particular set of cells. There is, however, a common feature to the patterning of the dorsal and ventral neural tube: in both halves inductive signals are initially expressed by non-neural cells (in the epidermal ectoderm dorsally and in the notochord ventrally). Then, through a process called *homeogenetic induction*, these signals are transferred to specialized glial cells at the midline of the neural tube (the roof plate dorsally and the floor plate ventrally).

Dorsoventral patterning is maintained throughout the rostrocaudal length of the neural tube: in other words, the strategies used to establish the dorsoventral pattern in the spinal cord appear also to control cell identity and pattern along the dorsoventral axis of the hindbrain, midbrain, and much of the forebrain.

Along the anterior-posterior axis the situation is somewhat more complex, with retinoids, FGFs, hedgehogs, Wnts and BMPs all proposed to function in different

locations or developmental windows. In some instances, the combinatorial actions of several factors acting on a single region or cell appear to establish regional pattern and neuronal diversity. In other cases, inductive factors, most notably Sonic hedgehog, can act as gradient signals, or morphogens inducing distinct neuronal subtypes at different concentration thresholds.

Rostrocaudal patterning begins at the neural plate stage and appears to be intimately linked with the process of neural induction itself. The neural tissue induced by follistatin, noggin, and chordin appears to express genes that are characteristic of forebrain but not of more posterior tissue. Thus, additional signalling pathways may be required for the induction of posterior neural tissue, which later gives rise to the midbrain, hindbrain, and spinal cord.

The anterior-posterior identity of cells in the neural plate is established by the combined action of different neural inducers and patterning signals. Collectively, these patterning signals progressively subdivide the neural tube along its rostrocaudal axis; as a result, neurons at the same dorsoventral position but at different rostrocaudal levels of the neural tube develop distinctive identities and functions.

From the anatomical point of view, one of the initial steps in the regionalization of the neural tube along its rostrocaudal axis is the subdivision into domains that give rise to the major regions of the CNS: the forebrain, midbrain, hindbrain and spinal cord. It has been demonstrated that the establishment of these regional identities employs mechanisms similar to those controlling segmentation in Drosophila embryo. In invertebrates, the final identity of each segment is defined by the differential expression of homeotic genes (see below for details). Homologues of many of these genes have been identified in vertebrates, and targeted-gene disruption studies have revealed their essential roles in the development of unique regions within the neural tube, as will be discussed in a following session of the thesis.

1.1.3 Neurogenesis

Neuronal progenitors within the neural plate and the neural tube are directed to differentiate into neurons and glial cells, the two major cell types of the nervous system.

Over the past decade, our understanding of neurogenetic mechanisms has improved to the point that it is now possible to define a core program of neurogenesis;

key insights into this issue come from studies performed in *Drosophila.* To understand the control of neuronal identity in vertebrates it is therefore necessary to review the key mechanisms and molecules used in *Drosophila.*

The selection of a single neuron from a large and initially uniform population of ectodermal cells in *Drosophila* involves a program of cell interactions that gradually restricts the fate of a cell.

The initial step in this program is the recruitment of a small cluster of ectodermal cells that acquire the potential to give rise to neuronal precursors. This region of the ectoderm is known as the *proneural region.*

Neuronal fate is decided by a process of signalling between adjacent cells in the proneural region. This process depends on interactions between two cell-surface proteins encoded by the neurogenic genes *notch* and *delta.* Both proteins span the cell membrane: delta functions as a ligand, and notch is its receptor. Initially all cells in the proneural region express both proteins at similar levels. The activation of notch by delta initiates a local feedback signal between adjacent cells that determines different levels of notch activation in those cells and consequently a different cell fate. Cells in which notch is activated to high levels are inhibited from acquiring a neural fate, whereas cells with relatively low levels of notch signalling become neurons.

It has become apparent that the notch-signalling pathway is highly conserved and also exerts a pivotal role in the control of neurogenesis in vertebrates.

The analysis of notch signalling in *Drosophila* has also led to the identification of intracellular proteins that regulate notch function. In particular, a cytoplasmic protein called numb binds to the intracellular domain of notch and in doing so inhibits notch signalling, thus promoting neuronal cell fate. Numb and many other proteins are concentrated at one pole of the proliferative neural precursors, and so they are segregated to one or both daughters, depending on the mitotic plane. As a consequence, the symmetry (or asymmetry) of a cell division may influence the nature of the progeny via symmetrical (or asymmetrical) intracellular partitioning of the same notch pathway components that are activated extracellularly by neighboring cells.

Studies of neurogenesis in Drosophila have also revealed many of the upstream regulators and downstream effectors of notch signalling. Most notably, transcription factors of the basic helix-loop-helix (bHLH) class have been shown to have central roles in defining groups of proneural cells. Members of the same gene families play

independent roles in directing the progression of neuronal differentiation once fate has been determined. Close relatives of these genes have been identified in vertebrates and shown to have strikingly similar functions in controlling the decision of progenitor cells to remain proliferative or to acquire neuronal or glial fates.

The basic mechanisms regulating neurogenesis in the vertebrate nervous system are similar to those operating in Drosophila. Vertebrate neural cells express both delta and notch proteins. Studies in Xenopus laevis, for example, have revealed that notch-delta interactions regulate neurogenesis as they do in flies.

Moreover, these studies have further revealed that not only molecules are conserved across phylogeny, but also entire programs of nerve cell differentiation.

1.1.4 Generation of neurons and glia from multipotential precursors

One of the most important aspects of the cellular organization of the nervous system is the presence of a large number of differentiated cell types that can mediate distinct functions. During development, differentiated cell types are generated from dividing progenitor cells. The mechanisms by which the principal cell types of the nervous system, neurons and glia, are generated from the progenitor population has been an area of active investigation for several years, and these studies have provided a number of important insights.

In the mammalian CNS, neurons are generated primarily in the embryonic period, while most glia are generated after birth (Jacobson, 1991). In mouse cerebral cortex, for example, neurogenesis starts around E12, peaks around E15, and finishes around birth (Bayer and Altaman, 1991; Jacobson, 1991). While the transient radial glia are present at early stages, macroglial [a detailed description of the glia cell types in the mammalian nervous system will be found in a following session of this thesis] production in the cortex does not start until midgestation and only at low levels (Abney et al., 1981; Skoff, 1990; Cameron and Rakic, 1991; Misson et al., 1991; Levison et al., 1993; Parnavelas, 1999). Cortical astrocytes are first detected around E16 and oligodendrocytes around birth, but the vast majority of both cell types is produced during the first postnatal month. The temporal separation of cell production allows the neuronal population to be well established before the glial system develops.

Despite the fact that the separate timing of neurogenesis and gliogenesis has been described for many years, the mechanism involved remains largely unknown. This issue becomes particularly intriguing if one consideres that CNS neurons and glia can arise from multipotential progenitor cells (Temple and Qian, 1996; Rao, 1999; Gage, 2000), although separate precursors for neurons and glia have also been described (Luskin et al., 1988; Davis and Temple, 1994;Williams and Price, 1995)

In most parts of the nervous system neurons and glia are generated in sequence from a progenitor cell population and at least a subset of these progenitors are capable of generating both cell types. Many of these multipotent progenitors can also produce daughter cells that retain the ability to give rise to both neurons and glia, and by analogy with the hematopoietic system these cells are referred to as neural stem cells.

Stem cells can generate differentiated progeny such as neurons and glia by either cell autonomous or non-cell autonomous mechanisms.

The sequential generation of neurons and glia in the cerebral cortex offers a good model system for evaluating the instructive and selective effects of extracellular factors, as well as cell autonomous mechanisms, in cell fate specification. It has, indeed, recently shown that cortical progenitor cells generates neurons before glia in a phased manner and a change in their behavior is critical for the timing process (Qian et al., 2000).

The generation of glial cells in the central nervous system is also controlled by secreted signalling factors.

The presence of a neuronal fate-inducing signal early in development and a glial fate-inducing signal late in development could then account for the observed temporal pattern of cellular differentiation.

The role of extracellular signals in regulating the development of glial cells has been extensively investigated, leading to the finding that the program of oligodendrocyte differentiation is controlled by platelet-derived growth factor (PDGF); the differentiation of progenitor cells into astrocytes is, in contrast, promoted by ciliary neurotrophic factor (CNTF). In particular, in the absence of PDGF, oligodendrocyte progenitors stop dividing and almost immediately differentiate.

More recently, Morrow et al. (2001) show that the growth factor FGF2 is an important signal for glial cell induction. These authors suggest a model: during neurogenic period the cortical progenitor is a multipotent cell that adopts a neural fate

unless it is exposed to a glial fate-inducing signal. FGF2 can act as a mitogen and glial fate-inducing signal for this cell, but during the neurogenic period most progenitors exit the cell cycle and differentiate into neurons because of low levels of extracellular FGF2. A developmental increase in FGF2 signalling induces a glial fate on remaining dividing progenitors such that all subsequent progeny are restricted to becoming glial cells.

Thus, in the central nervous system extracellular factors induce multipotent progenitors to adopt a specific cell fate and might mediate the sequential generation of neurons and glia throughout the nervous system.

1.2 NEOCORTICAL DEVELOPMENT

1.2.1 Construction of the mammalian cerebral cortex

The development of the cerebral cortex consists of a series of intriguing processes that lead to the structure that is vital for integrating sensory inputs and for cognitive and behavioral functions. Indeed, the neurons of the cerebral cortex are organized in two dimensions relative to the cortical surface. Tangential to this surface, different areas subserve a variety of functional modalities. Each areas is characterized by a set of distinctive features (Brodmann, 1909) and, most importantly, by the pattern of both afferent and efferent axonal connections. In the perpendicular dimension, the cortex is composed of six sheets or layers, each of which is defined by the density and the morphology of its constituent neurons.

The cerebral cortex originates from the wall of the neural tube as an extension of the two telencephalic vesicles. During the earliest stages of the telencephalon development, the wall of the neural tube shows a homogeneous structure (Bayer and Altman, 1990): the neurons of the cerebral cortex are generated from a sheet of dividing germinal cells that lines the embryonic ventricular system and is called neuroepithelium or *ventricular zone.*

The neuroepithelium is composed by columnar cells oriented perpendicular to the ventricular surface. Those cells are anchored by thin cytoplasmic processes to the inner and the outer membranes of the neural tube. Thus, the nuclei of the cortical progenitor cells move within the cytoplasm and undergo a series of elevator movements that correlate with their progression through the cell cycle (Sauer, 1935). During DNA

synthesis, or S phase of the cell cycle, the nuclei of the germinal cells move away from the ventricle within the cytoplasm and aggregate some distance from the ventricle in a band called *synthetic zone*; after DNA synthesis, the nuclei descend toward the ventricle in the *mitotic zone* to start the configuration of mitotic spindles. This nuclear movement is called *interkinetic nuclear migration* (figure 1.4).

As development proceeds, the neural wall of the telencephalon loses its homogeneous structure and fetal lamination evolves: once the progenitor cells left the cell cycle, the immature neurons migrate out of the ventricular zone to form the cortical plate, which eventually becomes the grey matter of the cerebral cortex. To reach the cortical plate, neurons migrate on radial glial cells that retain contacts with both the ventricular and pial surfaces.

As soon as immature neurons start to migrate out, a transitional field appears. This includes the subventricular zone (SV) and the intermediate zone (IZ), where differentiating cortical cells translocate before migrating to outer regions.

The first layer to form is a poorly packed layer of neural cells, called the primordial plexiform layer (PPL) or *preplate* (Bayer and Altman, 1991); this is composed by a superficial plexus of corticopetal nerve fibers and the earliest generated neurons, including the Cajal-Retzius and the prospective subplate neurons. Subsequently, the preplate is split into two by the insertion of later-generated neurons that form the cortical plate proper. Therefore, the preplate is split into a superficial layer the *marginal zone*, in which the Cajal-Retzius neurons differentiate, and the subplate (figure 1.5). During development, the thickness of the cortical plate progressively increases. Within the cortical plate, neurons become organized into well-defined layers; the final position of a cortical neuron, and therefore its final laminar position correlates with the birthday of the neuron. Cells that migrate from the ventricular zone and leave the cell cycle at early stages give rise to neurons that settle in the deepest layers of the cortical plate. In contrast, cells that leave the ventricular zone and exit the cell cycle at progressively later stages migrate over longer distances, overcome the early-born neurons, and settle in more superficial layers of the cortex. Thus, the layering of neurons in the cerebral cortex is established in an inside- first, outside- last manner (Rakic, 1978; McConnell, 1988). This inside-out pattern of neurogenesis produces

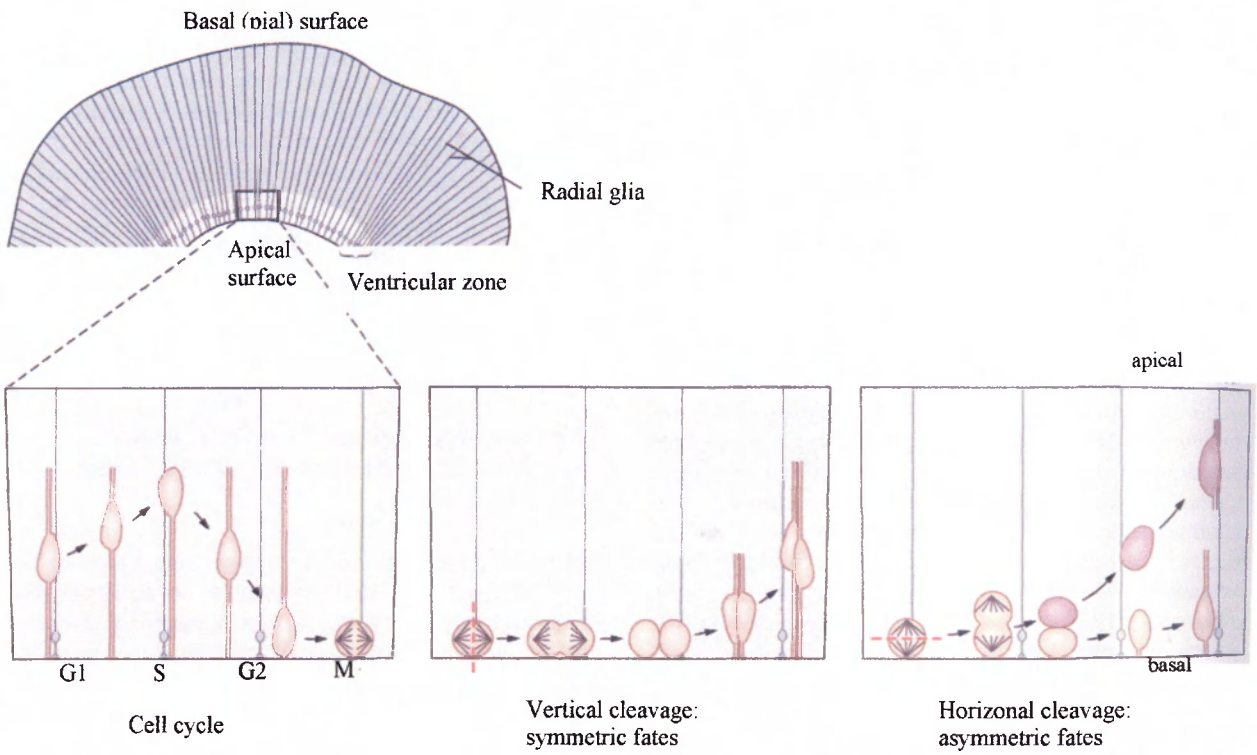


Figure 1.4

Interkinetic nuclear migration and models for symmetric and asymmetric division of neuronal precursors.

During G1 phase of cell cycle, nuclei rise from the inner (apical) surface of ventricular zone. During the S phase, the nuclei reside in the outer (basal) third of the ventricular zone. During G2, the nuclei migrate apically, and mitosis occurs when the nuclei reach the ventricular surface. The vertical cleavage (which is perpendicular to the ventricular surface) of the progenitor cell generates two similar daughters that retain their apical connections. Following mitosis the nuclei of both cells re-enter the cycle.

The horizontal cleavage (parallel to the ventricular surface) produces an asymmetric division in which the apical daughter retains contact with the apical surface, and the basal daughter loses its apical contact. The basal daughter migrates away from the ventricular zone and later becomes a post mitotic neuron. (From Chen and McConnell, 1995)

neurons within a given layer that share similar birthdays as well as common functional properties and connectivity.

As development proceeds, the thickness of the cortical plate progressively increases (figure 1.5). Both the transitional field and the cortical plate develop at the expense of the neuroepithelium according to specific spatial and temporal gradients. In particular, two major neurogenetic and morphogenetic gradients can be observed: one progressing to posterior, and a second progressing ventrolateral to dorsomedial.

1.2.2 Neuronal fate

The laminar destination of a cortical neuron is determined in the ventricular zone during the mitotic phase of the progenitor's cell cycle before the onset of migration into the cortical plate. Similarly, the commitment to a particular neural cell type also appears to be established in the ventricular zone, although certain cortical features such as peptide expression or morphological characteristics require local environmental interactions for proper development.

Early in neural development the progenitor cells that give rise to neurons of the mature cerebral cortex proliferate rapidly and give rise to additional progenitors, expanding the population of neural precursor cells. At later stages, however, progenitor cells alter their program of cell division and give rise both to neurons and additional progenitor cells. At still later stages they generate only neurons.

The orientation of the plane of cleavage of cortical progenitor cells, and consequently whether the division is symmetric or asymmetric, correlates with the fate of the cells. Cells that undergo vertical cleavage -in a plane that is perpendicular to the ventricle surface- divide symmetrically and generate two similar daughter cells that remain in the ventricular zone (figure 1.4). As a consequence, these cells undergo additional rounds of proliferation, expanding the population of progenitor cells in the ventricular zone. Cells that undergo horizontal cleavage -in a plane parallel to the ventricular zone- appear to divide asymmetrically, generating two daughter cells that have distinctive morphologies and migratory behaviors. The basal daughter cell loses its attachment to the ventricular surface of the cortex and migrates out of the ventricular zone to give rise to a young neuron. The apical cell, however, remains in the ventricular zone and undergoes further rounds of cell division (figure 1.4).

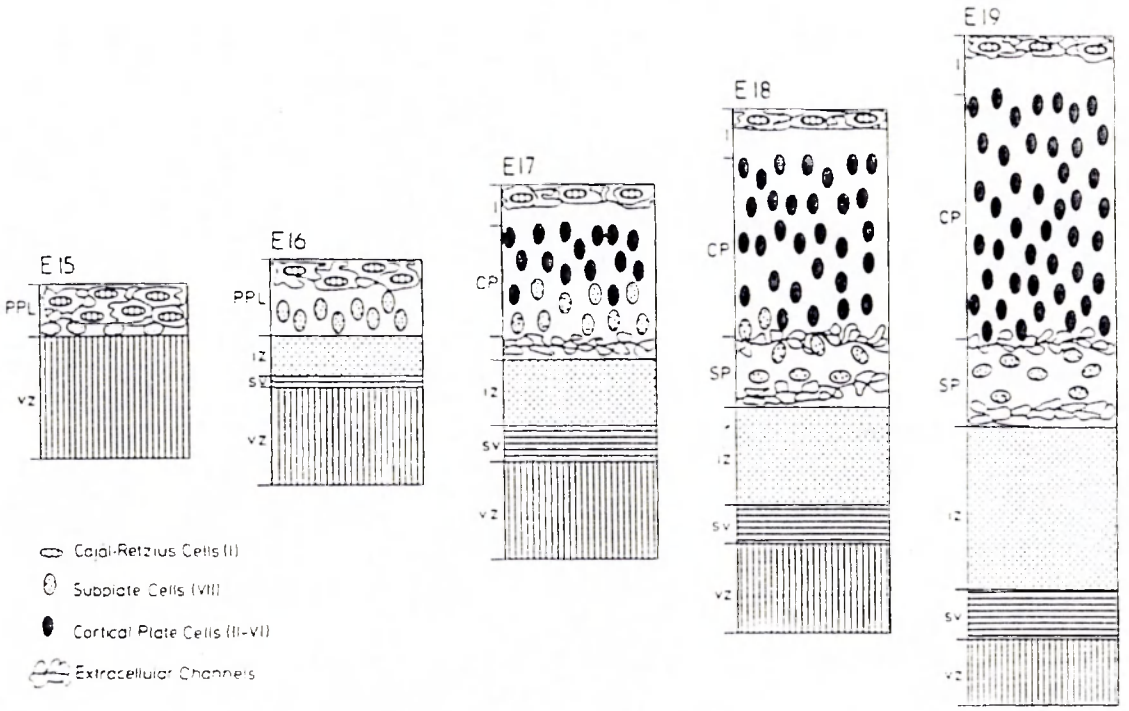


Figure 1.5

Development of cerebral cortex in rat, from E 15 to E19. (In mouse the same processes take place between E13 and E17). At the beginning of neurogenesis (E10, in mouse), the cerebral cortex consists of only the germinative neuroepithelium or ventricular zone (VZ). The first cellular population that appears above the ventricular zone is the primordial plexiform layer (PPL) or pre-plate (E15 in rat, that corresponds to E13 in mouse development). From E16 to E19 the PPL is progressively split into two layers, the most superficial marginal zone and the deepest subplate (SP). Between the marginal zone and the subplate postmitotic neurons accumulate to form the cortical plate (CP). In CP oldest neurons are located the deeper layers, whilst youngest neurons are in the more superficial ones.

It is interesting to note that prior to the generation of postmitotic neurons, most progenitor cleavages are vertical. The frequency of horizontal cleavages increases as the rate of neurogenesis increases late during development. This suggests that vertical cleavages are symmetric and proliferative whereas horizontal cleavages are asymmetric and neurogenic.

This notion is supported by the localization of mammalian homologs of fly Notch and Numb. The Notch 1 protein is localized to the basal pole of mitotic ventricular zone progenitor cells in ferrets (Chenn and McConnell 1995). In corresponding cells in mice, Numb protein forms an apical or apical lateral crescent (Zhong et al. 1996). Thus, the two proteins will be distributed to both daughters in a vertical cleavage. In a horizontal cleavage, Notch will be segregated to the basal daughter whereas Numb will stay in the apical daughter, therefore generating two molecularly distinct daughters

However, it should be mentioned that in contrast to the basal localization of Notch 1 immunoreactivity in ferret mitotic ventricular zone progenitor cells, Notch 1 immunoreactivity has been observed to be localized to the apical cortex of neural stem cells of adult rat CNS and that Notch 1 was found distributed around the entire cortex of dividing progenitors during mouse cortical neurogenesis (Zhong et al 1997, Johansson et al 1999).

Whether these results reflect species-specific or Notch 1 isoform-specific localization remains to be determined. In contrast to the apical localization of mouse Numb in mitotic ventricular progenitor cells, a recent study reported a basal localization of a chick Numb homolog in mitotic avian neuroepithelial cells (Wakamatsu et al 1999). The discrepancy between this result and the reported apical localization of the mouse Numb homologue (Zong et al 1996) could be due to differences between species or differences in the localization of Numb specific isoforms.

During development, the cells of the neural tube generate the enormous variety of neurons that will populate the central nervous system of the adult. Phenotypically diverse neurons must be produced, organized into functional units, and interconnected through the formation of specific axonal and synaptic contacts. These processes ultimately generate precisely organized neuronal circuits that underlie both simple and complex behaviors.

There are two general mechanisms that contribute to the determination of specific neuronal fates. One is the inheritance by a cell of a restricted developmental potential from its parent or ancestor, in other words the determination of the cell fate by cell lineage. The second mechanism implies that neuronal precursors or their progeny are multipotent, that is, the cells may develop along a variety of possible pathways, and the particular pattern chosen results from interaction between cells and their local microenvironment. The environment is thought to provide instructive influences that actively signal or induce the production of specific neural phenotypes.

The analysis of lineage relationships among cortical cells has proven much more complex than in other regions. Studies using retroviral lineage tracers suggest that clonally related cortical cells can spread extensively throughout the cortex, with clones dispersed even among distinct areas. This interpretation is supported by the direct demonstration that neurons can migrate tangentially within the intermediate zone and by evidence suggesting that progenitor cells disperse laterally within the ventricular zone. The tangential dispersion of both the progenitors and post-mitotic neurons means that a clone cannot be accurately defined as a spatially restricted cluster of cells.

1.2.3 Radial migration of neural cells

In the cortex, as in other regions of the developing central nervous system, neurons arise in a transient layer, the ventricular zone, and then migrate to their laminae before or as they differentiate.

The migration of neuroblasts and neurons is dependent on radial glial cells (Rakic, 1971, 1972, 1978; Sidman and Rakic, 1973). Several studies performed by using both in vitro and in vivo systems have demonstrated that 80-90% of the billions of cortical neurons migrate along radial glia cells (reviewed in Hatten, 1999). During development these cells maintain their contacts with both the ventricular and the pial surfaces of the developing neural tube. Therefore, as the wall of the neural tube thickens with the continued divisions of cells in the ventricular layer and the accumulation of neurons in the cortical plate, the radial glial cells become extremely elongated.

Glia-guided migration, indeed, seems to be the primary mechanism by which cortical neurons achieve their final destination. These phenomena imply the existence of

signals on or near radial glia that first promote migration of neuroblasts in appropriate directions, and then arrest movement at appropriate locations.

A number of neuronal and glial receptor systems have been implicated in the directed migration of CNS neurons along glial substrates (reviewed in Pearlman et al., 1998). Neuregulins , which are secreted growth factors, are produced by neurons in both the developing cerebellum and cerebral cortex, whereas their receptors (erbB2, erbB3 and erbB4) are expressed by both neurons and glia (Rio et al., 1997;Anton et al., 1997). Experimental evidence indicates that neuregulins affect neuronal migration as well as the growth and differentiation of radial glia (Rio et al., 1997; Anton et al., 1997).

It has been shown that diffusible signals from the young neurons induce the extension of processes by these glial cells. Among these, RF60 is a neuronal protein that is abundant in the mid-gestational period, when migration is robust, decreasing in later periods when migration wanes, and undetectable in the adult, after the program of migration has established the neuronal laminae (Hunter and Hatten, 1995).

The neuronal protein astroactin (ASTN) has been the most extensively studied receptor system in functional assay of glial-guided neuronal migration. Antibody perturbation assays demonstrate that ASTN provides a ligand for neuronal binding to the glial fiber during migration. Although other surface receptor systems can support neuronal movement, removal of ASTN reduces the rate of neuronal migration by approximately 60%.

Studies on mutant mice with brain malformations have provided another approach to the discovery of genetic loci that contribute to neuronal migration in developing brain. Two of these mutants, *weaver* and *reeler*, have long been assumed to be models for cell migration.

In weaver mice, granule cell precursors in the cerebellar cortex fail to migrate along glial fibers and die in ectopic positions. In vitro studies (Gao et al., 1992) and the production of mouse chimeras (Goldowitz, 1989) have demonstrated that the weaver gene acts in immature neurons.

The *reeler* mutation, first described over 45 years ago, produces an abnormal architecture in many parts of the brain, but the disruption is most dramatic in the cerebral and cerebellar cortices (Caviness and Rakic, 1978; Goffinet, 1984). In the *reeler* cortex, although cell generation and the initial phases of migration are normal, the preplate is not split into the MZ and the subplate by the forming cortical plate.

Cajal-Retzius neurons remain at the top of the undivided preplate or "superplate". Cortical plate cells accumulate beneath the superplate in a highly disordered, non-laminar fashion.

The reeler gene codes for an extracellular matrix (ECM)-like protein, termed *reelin*, that may interact with other adhesive proteins and mediate cell adhesion. Reelin is produced by Cajal-Retzius neurons in the upper preplate and in the marginal zone but not by cells in the subplate. Reelin is neither produced by, nor distributed along radial glia, meaning that this protein is likely to provide a stop signal to cells at the end of their journey. Without reelin in the marginal zone, cortical neurons might reverse direction, then migrate downward and accumulate beneath the preplate.

In the last year, three independently discovered mutant mice with reeler-like phenotype have all been found to have mutation in the same gene, named *mdab1*. This gene is expressed by migrating cortical neurons that will make contact with reelin. It is therefore reasonable that mdab1 is part of a cascade that responds to reelin.

To date, mutations in other genes, such as cdk5 and p35, have been shown to affect cortical lamination with patterns different from reeler.

The study of the mechanisms regulating cortical migration is strongly enhanced by the fact that many severe cortical malformations in humans, such as Lissencephaly, are caused by abnormal neuronal migration and have a genetic origin

In the mammalian telencephalon, radial glia disappears by the end of the process of migration. Several studies performed in a variety of mammalian species have suggested that these cells transform into astrocytes (reviewed in Rakic, 1995).

The development of neuronal and glial lineages in the mammalian neocortex has been the subject of investigation since the 1980s. In particular, the prevailing view has for long time been that neurogenesis and gliogenesis are distinct events during corticogenesis; moreover, lineage analyses have documented that, apart from relatively few exceptions, neurons and glia arise from separate lineages (Parnavelas, 1999, 2000).

Thus, until recently, it has been widely believed that the germinal ventricular zone is primarily composed of neuroepithelial cells that give rise to distinct populations of neurons and glia, including radial glia. However, this concept is gradually being eroded in the light of the emerging evidence that ascribes a new and radically different role for radial glia cells in the developing cortex.

Several recent reports have proposed that radial glia may be potential precursor cells of neurons (Chans-Sacre et al., 2000; Malatesta et al., 2000; Hartfuss et al., 2001; Miyata et al., 2001; Noctor et al., 2001) (fig.1.6). Together these studies suggest that radial glia, while able to self renew, also generate neurons during early corticogenesis and astrocytes at later stages.

Movement along radially oriented glial cells is not the only mechanism by which migrating neurons are guided to their final destination (Rakic, 1995). Many neurons migrate through regions of the central nervous system in which there are no radial glial cells. Indeed, radial migration does not account for the significant tangential dispersion of clonally related cortical neurons: both postmitotic neurons and progenitors may contribute to this dispersion. First, a significant proportion of postmitotic neurons generated in the ventricular zone migrate tangentially within the intermediate zone (O'Rourke et al., 1995); second, tangentially oriented, postmitotic neurons are present within the ventricular zone and subventricular zone and can migrate long distances within these regions (O'Rourke et al., 1997); third, neuronal precursors also move tangentially within the ventricular zone (Fishell et al., 1993; Reid et al., 1995).

Finally, although it has been for long believed that neocortical neurons originate only from the cortical proliferative zone, in recent years it has been demonstrated that a population of neurons moves tangentially into neocortex from proliferative zones that are actually outside the neocortex. In particular, those neurons are GABA-expressing cells originated in subcortical regions.

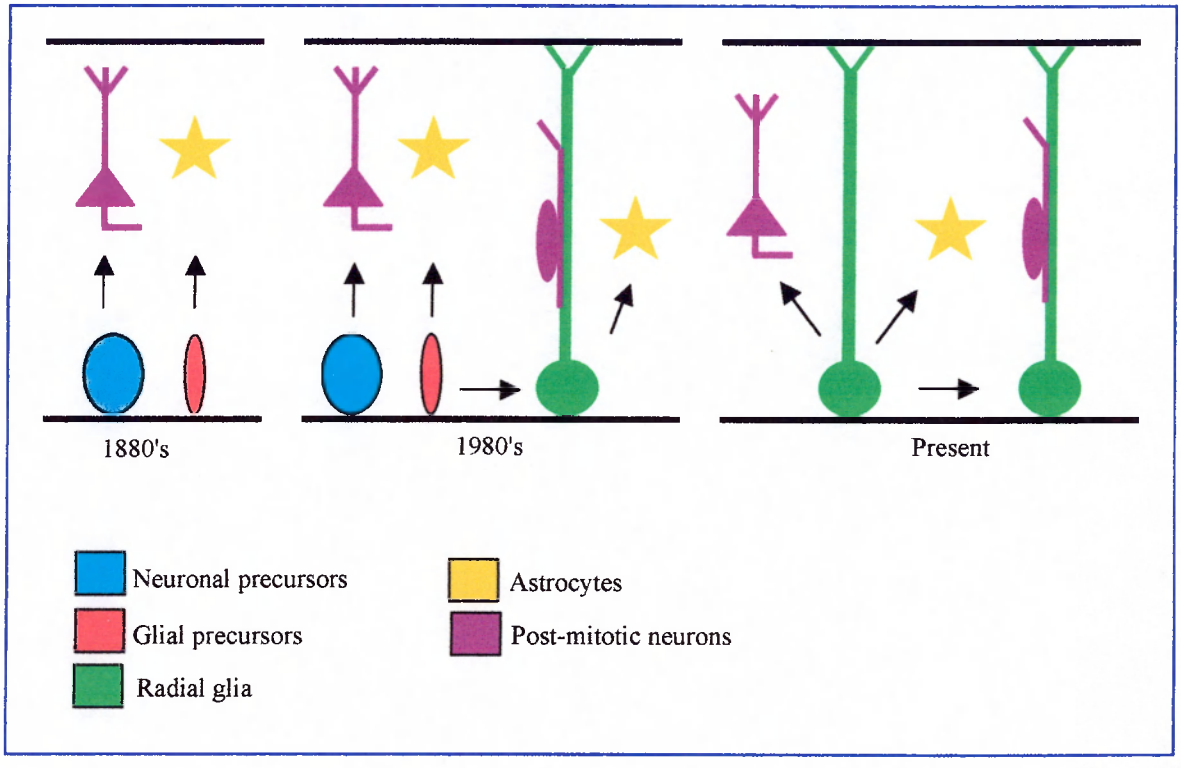
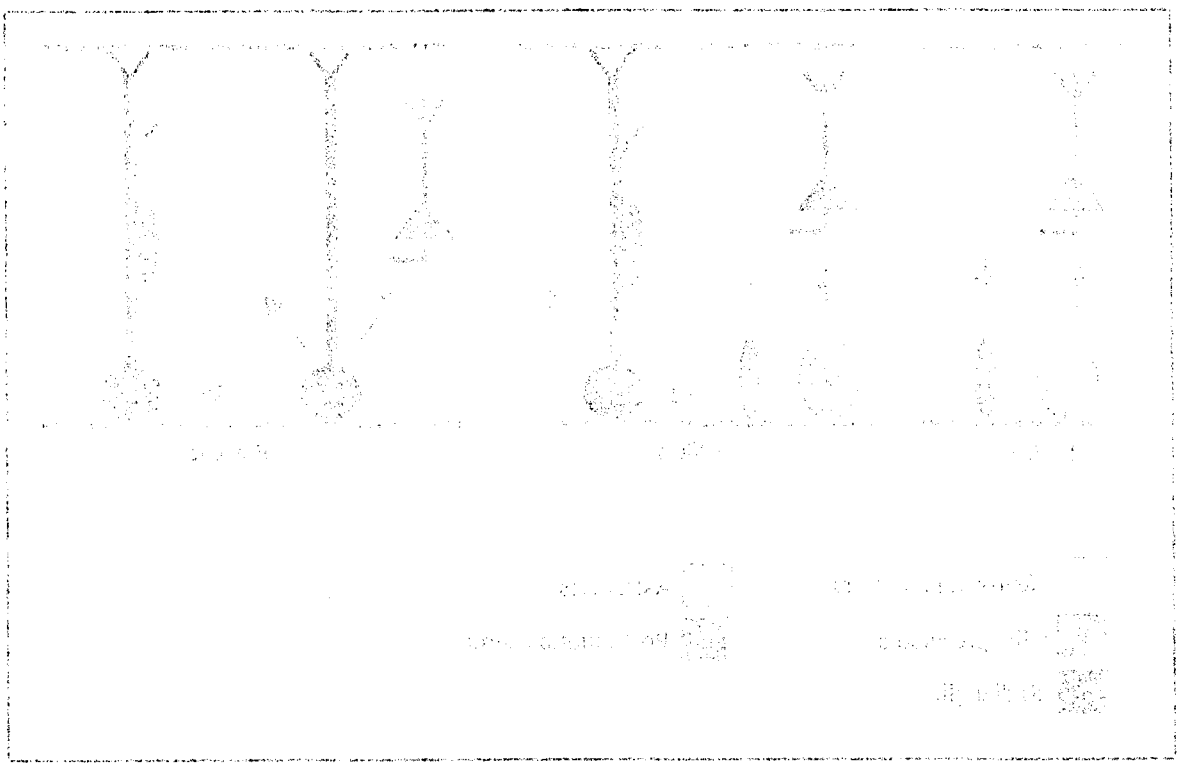


Figure 1.6

Three models illustrating proposals on how cell diversity is generated in the mammalian cerebral cortex from the 1980s to present.



1.2.4 Migration of interneurons from basal forebrain to neocortex

The neocortex of the mammal derives from the telencephalic vesicle in a domain identified by the expression of a number of transcription factors. The vesicle is later subdivided into regions known as pallium and subpallium whose derivatives form the cerebral cortex and the basal ganglia in mammals. The subpallium expands into the ventricle to form the ganglionic eminence, which is later separated into the medial and the lateral subdivisions (MGE and LGE, respectively), by an invagination. The importance of the boundaries between these morphological subdivision is emphasized by the expression patterns of a number of genes: Gli3, Ng1 and ng2 in cortex, Mash1, Dlx1 and Dlx2 in both ganglionic eminences, and Nkx2.1 in the MGE alone (Wilson and Rubenstein, 2000).

Several studies have demonstrated that GABAergic interneurons invade the neocortex from the LGE and the developing striatum (De Carlos et al., 1996; Anderson et al., 1997). The number of GABAergic interneurons throughout the neocortex is dramatically reduced by separating the neocortex from the underlying LGE/striatum during early embryonic development, suggesting that the LGE is the major source of neocortical interneurons (Anderson et al., 1997). This process is also disrupted in the absence of the transcription factors Dlx1 and Dlx2 (Anderson et al., 1997), and may be regulated by neurotrophins.

Thus, cell migration in the developing telencephalon can broadly be divided into two categories, radial and non-radial. Neocortical cells that are born in the cortical ventricular zone use radial glia as a scaffold upon which to migrate to the developing cortical plate. Those radially migrating cells primarily give rise to the projection neurons that express the neurotransmitter glutamate (Parnavelas, 2000). In contrast, tangentially migrating cells that are generated in the ganglionic eminence give rise to the majority of cortical interneurons (reviewed in Corbin et al., 2001); those neurons migrate from basal to dorsal telencephalon along the fibers of the corticofugal projection system and use GABA as neurotransmitter (Parnavelas, 2000; Denaxa et al., 2001). To date, the MGE has been shown to be source of cortical interneurons as well as striatal interneurons. On the other hand, the LGE seems to be source of interneurons in the olfactory bulbs (Wichterle et al., 1999).

There is still much to be learned concerning the source and the cell types that undergo tangential migration; for example, numerous studies support the idea that most interneurons are derived ventrally, but it is not yet clear if other cell types, such as glutamatergic neurons or glial cells, are generated from these regions. Moreover, the question why specific cell populations need to be born long distance from their final destination is still open.

1.2.5 Specification of cortical areas

Regionalization of the cerebral cortex occurs during development by the formation of anatomically and functionally distinct areas. Indeed, the cerebral cortex in humans can roughly be divided into 52 distinct regions (figure 1.7). Areas are distinguished from one another by major differences in their cytoarchitecture and chemoarchitecture, and their input and output connections.

It has been assumed that the specification and differentiation of neocortical areas is controlled by interplays between genetic (intrinsic) and epigenetic (extrinsic) mechanisms (figure 1.7). However, until recently, most experimental evidence has implicated extrinsic mechanisms, in particular the influence of thalamocortical axons (TCAs) (Chenn et al., 1997). Only in the last two years, evidence for the genetic regulation of arealization has begun to emerge (Bishop et al., 2000; Mallamaci et al., 2001; Zhou et al., 2001).

In particular, it has recently been proposed that the processes of cortical regionalization and specification are driven by two phases. An early regionalization step takes place before axons coming from the thalamus reach the cortex, where neurons establish their regional identity by regulating their gene expression in a cell-autonomous way. Subsequently, a second specification step occurs upon the arrival of thalamocortical projections, and this might carry on extrinsic factors and mechanisms allowing the neo-formed cortical areas to be maintained and redefined.

Numerous studies indicate that late differentiation of anatomical features that distinguish cortical areas depend upon TCA input. Indeed, TCA projections exhibit area-specificity throughout development and the gradual differentiation of areas within the CP parallels the elaboration of the TCA projections.

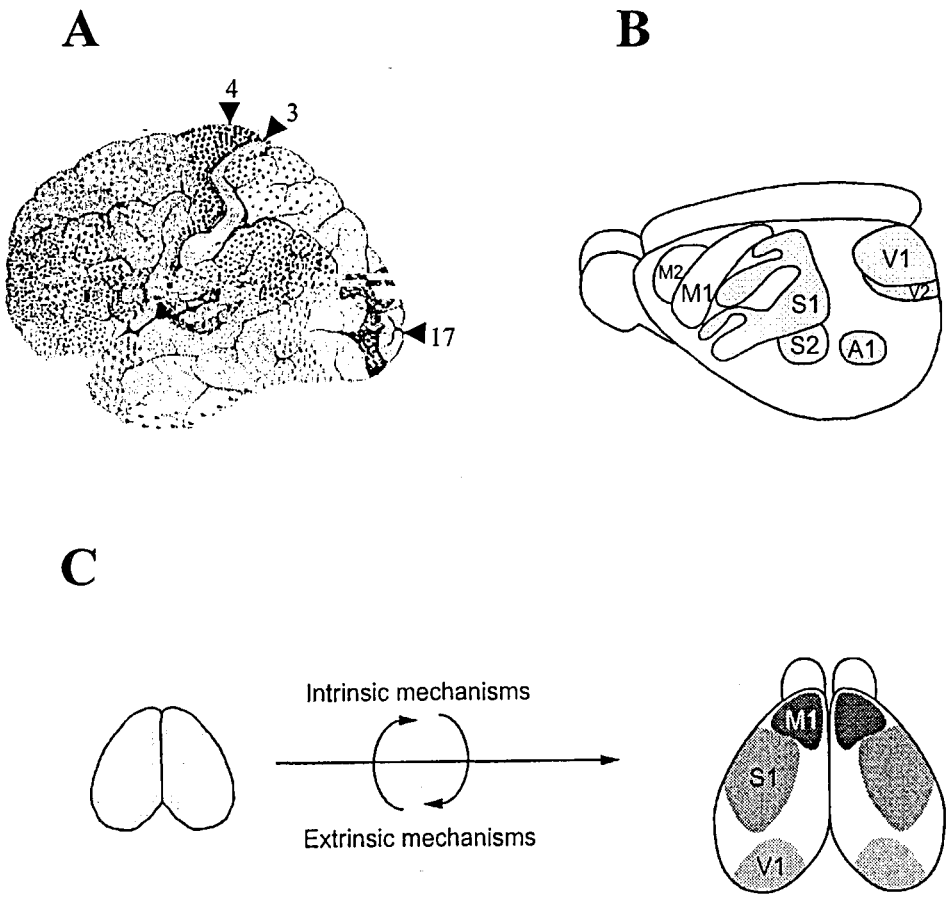


Figure 1.7

Organization of the neocortex into areas. A, areas of the human cerebral cortex as defined by Brodman. The lateral surface of the human cerebral hemispheres shows a number of cytoarchitectonally distinct areas. Here, three regions are evidenced: the primary visual area or V1 (area 17) at the caudal pole, the primary somatosensory area or S1 (area 3) in the middle and just rostral to it, the primary motor area or M1 (area 4). Subsequent analyses confirmed that these areas are also functionally and connectionally distinct. B, selected areas of the rat neocortex, showing A1, V1, V2, S1, S2 (secondary somatosensory area), as well as M1 and m2 (the secondary motor area). C, development of neocortical areas. Initially, the neocortex lacks many features that distinguish areas in the adult. Area-specific features gradually differentiate within it, by a process controlled by mechanisms both intrinsic and extrinsic to the neocortex.

The molecular control of area-specific targeting of CAS within the neocortex has still to be elucidated. Nevertheless, area-specific TCA targeting seems to be dependent on neural activity (Catalano and Shatz, 1998), and in particular that of SP neurons (Molnar, 2000; Ghosh et al., 1990). Recently, members of the cadherin family of cell adhesion molecules and of the Eph family of receptor tyrosine kinases and their ephrin ligands, have been suggested to be good candidates for the molecular control of TCA pathfinding.

However, several experiments suggest that early cortical regionalization does not require extrinsic influences. On the other hand, several genes have been shown to be regionally expressed in the cerebral cortex before thalamic input has penetrated the cortical plate (reviewed in Rubenstein et al., 1999).

To date, until a few years ago, the genetic control of cortical arealization was just an hypothesis or a model based on the description of genes, encoding transcription factors, nuclear receptors, cell adhesion molecules and so on, expressed in graded or restricted patterns within the CP or the VZ prior to the arrival of TCAs into the neocortex. The study of *Gbx2* and *Mash1* mutant mice, that fail to develop TCA projections, confirmed that the expression of potential arealization regulatory genes is unchanged in mutants, indicating that these patterns are established by mechanisms independent of TCAs (Tuttle et al., 1999; Nakagawa et al., 1999; Miyashita-Lin et al., 1999).

Genes that regulate arealization presumably confer area identities to cortical cells and regulate the expression of axon guidance molecules that control the area-specific targeting of TCAs. In particular, genes proposed to regulate specification of area identity are Emx2 and Pax6, that are supposed to cooperate during arealization processes by acting in opposite gradients, and COUP-TF1 (Bishop et al., 2000; Mallamaci et al., 2000; Zhou et al., 2001).

It has to be also reported that recent studies have begun to define candidate patterning centres and signalling molecules (FGF8, Shh, BMPs and Wnts) that act early in development to establish and maintain the graded expression of regulatory genes such as Emx2 and Pax6 across the neocortical VZ.

Therefore the present scenario of the arealization program is complex, involving extrinsic, intrinsic and signalling factors (figure 1.7) that act presumably in concert on one hand, but with differential patterns of expression in each area, on the other.

1.3 HOMEOBOX GENES IN DEVELOPMENT

A crucial role in the specification of the CNS and its subsequent subdivision into anatomically and functionally distinct neuronal domains is played by the so-called regulatory genes.

Regulatory genes exert their function by controlling the expression of other genes lying hierarchically downstream of them, and then called target genes; regulatory genes code for transcription factors, i.e. nuclear proteins able to recognise and bind certain cis-acting DNA sequences and modulate the expression of the corresponding target genes.

Several of these genes contain a DNA motif termed *homeobox* and are thus called *homeogenes.* The homeobox is a 180bp nucleotide sequence coding for a 60 amino acid residue protein domain called *homeodomain.* The homeodomain is able to recognise and bind specific DNA sequences. Proteins containing the homeodomain (*homeoproteins*) are transcription factors that act as activators or repressors of groups of downstream target genes.

The first homeobox genes were identified in the early 1980s in the fruit fly *Drosophila melanogaster.* Decades of genetic analysis have led to the identification of several regulatory genes necessary for crucial stages of the developmental of Drosophila, such as the establishment of the anterior-posterior axis and its patterning in a succession of appropriate anatomical regions or body segments.

Earlier studies have shown that there are mainly three classes of genes that are responsible for Drosophila development: maternal genes, segmentation genes and homeobox genes (Akam, 1987; Ingham, 1988). These genes are ordered into a hierarchy, by which specific genes organise individual regions of the embryo in progressively finer detail.

The function of maternal genes, that are already active in the body of the mother, is to introduce into the unfertilised egg all the molecules necessary and sufficient to specify the anterior and posterior extremities, as well as the dorsal-ventral polarity, of the future organism. After fertilisation, segmentation genes control subdivision of the fly embryo in a numerically and positionally precise succession of potential body segments. Once those genes have operated, the genes of the third category finally provide molecular information to specify the identity of the various

segments. Through the action of the homeotic genes, every segment along the body of the fly acquires its full endowment of anatomical structures, including appropriate appendages, and is put in the position to deploy its specific functionality. Indeed, the name homeobox derives from findings that mutations in some of those genes lead to homeotic transformations in which one body structure develops in place of another.

Interestingly, earlier analyses have defined the genetic logic by which the body plan of Drosophila is controlled: the genes responsible for the body plan are clustered together in the genome, in two contiguous loci called collectively HOM-C, meaning homeotic complex. The linear arrangement of the genes on the chromosome corresponds to the domains of expression and function of the genes in the embryo (figure 1.8): the gene at the most 3' is expressed most anteriorly, and the gene at the most 5' more posteriorly.

The study of vertebrate genes partially homologous to regulatory genes controlling the development of the Drosophila embryo has provided invaluable information about the genetic control of positional values in development. Many of these genes are homeobox genes, controlling cell identity in specific regions or segments both in invertebrates and vertebrates (McGinnis and Krumlauf, 1992). There are several families of homeobox genes. These include the *Hox* genes (Krumlauf, 1994) which are the vertebrate homologues of Drosophila homeotic genes belonging to the HOM-C complex. As in flies the genes of the HOM-C complex are known to control segment identity along the major rostro-caudal body axis, in many embryonic contexts the vertebrate *Hox* network (figure 1.8) is part of an evolutionary conserved mechanism for specifying regional differences along the embryonic body axis (Krumlauf 1993; McGinnins and Krumlauf, 1992). In particular the Hox genes provide positional cues for the developing hindbrain and specific regions along the spinal cord.

In mouse and human genomes there are four homeobox gene clusters (*Hoxa*, *Hoxb*, *Hoxc*, and *Hoxd*) located on different chromosomes (figure 1.8). The HOM-C complex in Drosophila and corresponding Hox clusters in mice are thought to have arise from a common ancestor of vertebrate and insect.

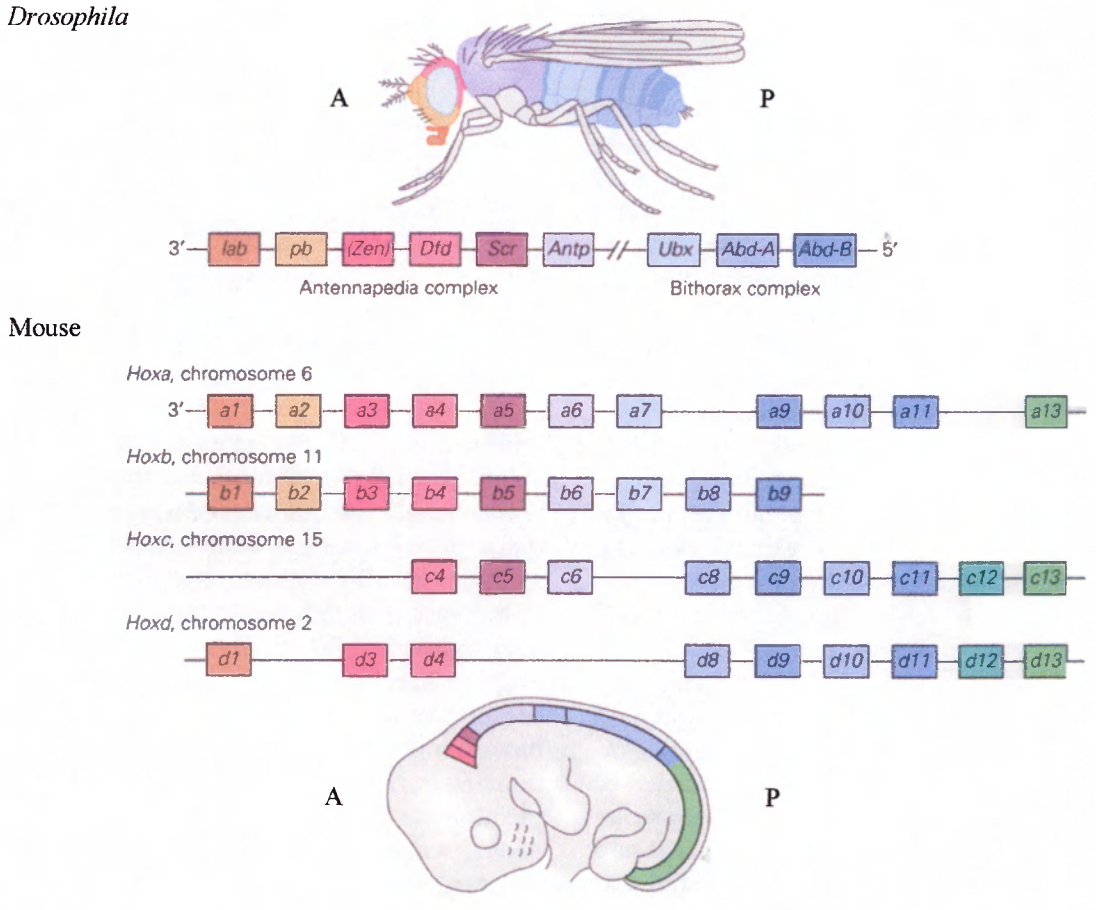


Figure 1.8

The clustered organization of the *Hox* genes is conserved in flies and mammals. The diagram shows the chromosomal arrangement of structurally related Hox genes in the mouse and HOM-C genes in Drosophila. The mouse has four Hox gene clusters, as do humans. In vertebrate the four clusters are located on different chromosomes. In both Drosophila and vertebrates these homeodomain proteins are involved in specifying regional identity along the anterior- posterior axis of the embryo.

1.4 SPECIFICATION AND DEVELOPMENT OF THE ANTERIOR REGION OF THE ANIMAL EMBRYO: FROM FLY HEAD TO MAMMALIAN FOREBRAIN.

The development of the anteriormost body domain corresponding to the head has remained relatively obscure in both invertebrate and vertebrate until the early 1990s (Finkelstein and Boncinelli , 1994 for review). Before the huge amount of genetic work done in the last decade, the nature of the anterior head segmentation in the insect embryo has been controversial and the genes that govern it were mostly unknown (Finkelstein and Perrimon, 1991; Cohen and Jürgens, 1991). In vertebrates the existence of compartments or segments in the forebrain and midbrain has been contested and the molecular mechanisms of pattern formation have not been determined.

A breakthrough has come with the identification of Drosophila genes and their vertebrate counterparts that appear to be critical for specification of the head and brain.

1.4.1 Development of the anterior head in Drosophila

In the Drosophila embryo, the head is considered as composed of a posterior and an anterior portion. In the posterior head three posterior or gnathal segments are morphologically distinct; whereas, the anterior or pre-oral head consists of an unsegmented region (the acron) and several cephalic segments whose morphology is more difficult to discern. The cephalic segments include (from anterior to posterior) the labral segment, one or more possible pre-antennal segments, the antennal segment and the intercalary segment.

More than a decade of elegant work has elucidated a genetic cascade specifying segments in the Drosophila trunk and involving a series of sequentially activated sets of genes. It has also supported the idea that the three gnathal segments of the posterior head are formed by genes in this hierarchy (Choen and Jürgens, 1991). On the other hand certain observations suggest that a different mechanism operates in the anterior head (Boncinelli and Finkelstein, 1994).

An important advance in understanding head formation has come with the identification of genes shown to be involved in controlling the anterior head specification and development. Among them, three genes have been shown to play

critical role in patterning the anterior head of Drosophila, namely *empty spiracles* (*ems*) (Dalton et al, 1989; Cohen and Jürgens, 1990; Walldorf and Gehring, 1992), orthodenticle (otd) (Finkelstein and Perrimon, 1990; Finkelstein et al, 1990), and buttonhead (btd) (Cohen and Jürgens, 1990). Mutations in each of these genes affect embryonic head development. The detailed analyses of each mutant phenotype (Finkelstein and Perrimon, 1990; Cohen and Jürgens, 1990) have shown that the absence of any one of these genes causes the loss of specific head structures, rather than their transformation into different structures. Moreover, the domains specified by these genes overlap, but are out of phase by one segment at their posterior border. *btd* mutant embryos lack the mandibular, intercalary and antennal segments; *ems* mutant embryos lack the lintercalary and antennal segments, and *otd* mutant embryos lack the antennal and a larger ill-defined preantennal region.

All these observations led to the proposal that these genes could function both as gap genes (to form groups of head segments) and as homeotic genes (to specify segmental identities). The overlap in their function and expression pattern further suggests that they could act in a combinatorial fashion.

1.4.2 otd and ems

otd and *ems* have been cloned and shown to contain a homeobox (Dalton et al, 1989; Finkelstein et al, 1990), suggesting that they act as transcription factors. They are expressed in broad stripes in the embryonic head before blastoderm cellularization and their expression is dependent on the maternal gene bicoid (Finkelstein and Perrimon, 1990).

The *ems* gene, named because it is required for the development of the tracheal system in abdominal segment 8, is expressed, at the blastoderm stage, in a fairly anterior circumferential stripe, that is under the regulation of *bicoid* gene product (Dalton et al 1989). Later during development, the ems expression becomes localised to specific head regions of the extended germ band of the embryo. When the gene is mutated, these segments are missing and defects in brain segmentation appear (Hirth et al., 1995). In order to achieve the specification of head segment identity, ems cooperates with *buttonhead* (*btd*). The same gene interaction is present in the trunk region of fly embryo, particularly in interneurons and ventral nerve cord (VNC), where ems is also

expressed and confer identity to trunk segments, but here its activity is prevented by Hox -cluster genes (Schoock et al., 2000). If the transcriptional networks and the genes implicated in the developmental control of ems-expressing embryonic districts are just starting to be elucidated, ems promoter regions and factors involved in its transcriptional are not yet determined.

At the blastoderm stage, *otd* is expressed in a circumferential stripe of cells at the anterior end of the egg (Finkelstein et al, 1990). This early domain of expression includes the precursors of the regions affected in the *otd* mutant embryos; indeed, in homozygous embryos, lethal *otd* alleles cause pattern deletions in the antennal and preantennal regions of the head (Wieschaus et al, 1984; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). In addition to its early role in the anterior region of the embryo, *otd* is required later in development: shortly after gastrulation begins, the gene is also expressed at the ventral midline of the embryo in neuronal, glial and epidermal precursors. Consistent with this, both neuronal and epidermal defects are seen in the ventromedial region of the *otd* mutant embryos (Wieschaus et al, 1983; Finkelstein et al, 1990; Klämbt et al 1991; Wieschaus et al 1992): in the CNS *otd* is required to specify medial neurons necessary for correct axonal guidance; in the ventral epidermis of the larva, the orthodenticle bands that mark the anterior margin of each embryonic segment are reduced in size and in type of pattern elements that they contain, from which the name *orthodenticle.*

The findings that vertebrate homologues of the Drosophila homeotic genes also function in axial patterning, have prompted a search for vertebrate genes related to the *btd*, *otd* and *ems.*

2.4.3 The vertebrate head: Otx and Emx

It has been shown that many genes responsible for, axial patterning have been conserved during evolution; a striking example, reviewed above, is seen in vertebrate Hox genes.

In flies, there is a developmental distinction between specification of the anterior head and the posterior head and the trunk: distinct sets of regulatory genes are involved in the specification of distinct body segments. This mechanism appears to be evolutionary conserved.

In vertebrate there seem to be a similar distinction between determination of the anterior head, composed of forebrain and head mesoderm, and of the trunk. Already in the first decades of the last century, Spemann (1938) proposed that in gastrulating amphibian embryo it is possible to distinguish between a head organiser, responsible for head formation, and a separate trunk organiser, responsible for body axis formation. In fact, if an early gastrula dorsal lip region has transplanted into another embryo, it gives rise to a secondary axis with head and brain structures; conversely, transplants of late gastrula dorsal lip regions give rise to trunk and tail structures. These observations can in turn be justified by either the fact that the two types of organisers are distinct individual entities or that they simply represent the two extremes of a continuum of opposing morphogenetic activities.

From the point of view of specific cellular morphogenetic movements, a clear distinction can be made in the frog embryo between the anterior head and more posterior body regions. In fact, the prechordal head mesoderm and the overlying forebrain are characterized by the absence of the powerful convergent-extension movements, taking place during gastrulation and neurulation, that are characteristic of more posterior region of the axis, such as the prospective hindbrain and spinal cord, as well as the corresponding axial mesoderm (Keller et al., 1992). According to this analysis, the decisional events leading to the specification of head versus trunk region must occur very early in development, possibly earlier that gastrulation itself.

Head specification was previously thought to have arisen independently in the vertebrate and invertebrate lineages.

By using Drosophila sequences, it has been possible to identify vertebrate homologues and cloned *Emx1* and *Emx2* (Simeone et al, 1992a), related to *ems* and *Otx1* and O*tx2* (Simeone et al, 1992b, 1993), related to *otd*. The four genes are then related to genes that, in Drosophila, are expressed during the formation of the most anterior part of the body; interestingly, in mouse they are expressed in extended regions of the developing rostral brain of mid-gestation embryo including the presumptive cerebral cortex and the olfactory bulbs.

The sequence conservation and the related expression patterns of the Ems and Otd gene families, by contrast with previous hypothesis, seems to suggest that the anterior patterning was established in a primitive ancestor of both flies and vertebrates.

1.5 THE VERTEBRATE EMX AND OTX GENES: THE GENE STRUCTURE AND THE EVOLUTION.

Studies of the Hox genes, of sonic hedgehog signalling and of many other genes involved in embryonic development have led to one of the fundamental insights in modern biology: molecules are conserved during evolution.

In particular, there is a considerable body of knowledge about the clusters of regulatory genes specifying the regional identity in the trunk of both invertebrates and vertebrates (Carroll, 1995). The HOX clusters have been extensively investigated in many different organisms. Comparative studies have revealed the common features of these clusters and their striking conservation along evolution from nematodes to mammals, suggesting a common origin in metazoan phyla as old as 600 My or even older (Wray et al, 1996). To date, homeodomain proteins have been found not only in metazoans but also in fungi and plants, leading to the idea that they arose early during the evolution of eukaryotes.

In the course of the evolution, the amino acid sequence of the homeodomain has been conserved to high degree. A striking sequence homology is found between Otx genes and their Drosophila cognates in the homeodomain. In fact, the homeodomain of the predicted mouse Otx1 and Otx2 proteins are similar to that of the fly protein, differing by only 3 and 2 amino acid residues, respectively (out of 60). The only other sequence similarity between *otd* and Otx genes however is limited to residues immediately flanking the homeodomain. More differences are found between Emx and *ems* genes: there are 11 and 8 amino acid residues different between *ems* and Emx1 and Emx2 respectively (figure 1.9).

Emx and Otx genes are highly conserved among the vertebrate species examined so far. In the case of Otx2, for example, between mouse and man there is a single conservative amino acid substitution (outside the homeodomain) out of approximately 300 residues (Simeone et al., 1993). There are no amino acid residue differences between mouse and human within the homeodomain, either for the Emx or for the Otx genes (figure 1.9). Instead, between mouse and *Xenopus* cognates there is a single amino acid substitution in the homeodomain of all the four genes; the *Xenopus* and murine Otx2 proteins have an overall sequence similarity of 95% (Pannese et al., 1995).

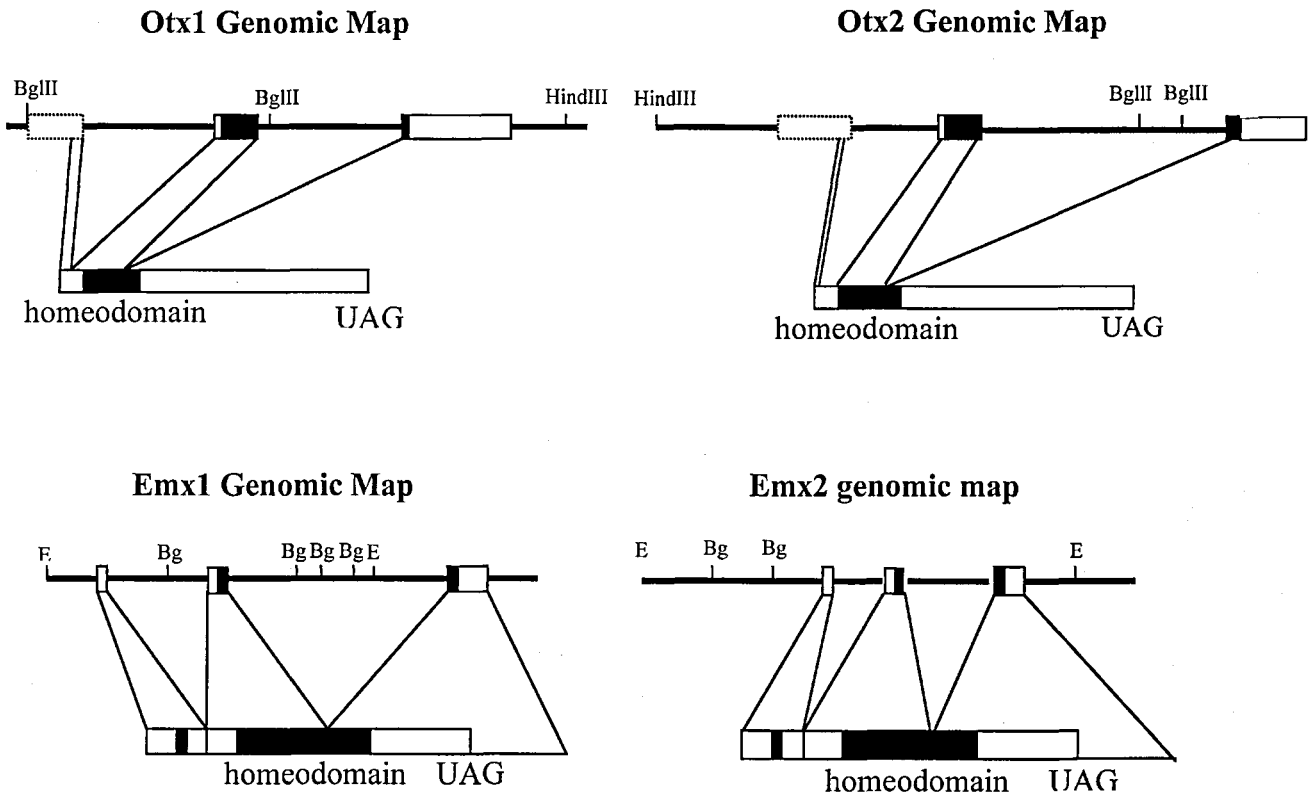
otd- and *ems*-related genes have also been isolated from chick (Bally-Cuif et al., 1995) and zebrafish (Mercier et al., 1995).

The four human genes, two EMX and two OTX, have also been mapped. OTX2 maps to chromosomal region 14q22; the OTX1 locus maps to 2p13; EMX2 maps to 10q26.1, and finally EMX1 maps to 2p14-p13 (Kastury et al., 1994).

As for the gene structure, human and murine Emx genes are composed of three exons; in both genes an intron is present between the exons containing the homeodomain and the homeopentapeptide, that is a short protein domain located upstream from the homeodomain (Dalton et al 1989) (fig. 1.8), and that is reported in several homeobox genes of Drosophila and in most vertebrate genes belonging to the Hox clusters (Boncinelli et al., 1991). An additional intron is present in both genes within the homeobox in the identical position, namely residue 44 of the homeodomain (fig. 1.9) (Simeone et al., 1992).

The structure of the mouse and human Otx 1 and Otx2 is similar. Those genes have the same exon-intron scheme: three exons and two introns. An intron is present immediately upstream of the homeodomain, and the exon upstream of this intron ends with the same motif, that possibly represents a divergent version of the conserved homeopentapeptide. The second intron is present within the homeobox in the identical position, namely between residues 46 and 47 of the homeodomain.

A



B

Ems	PKRIRTAFSP SQLLKLEHAF ESNQYVVGAE RKALAQNLNL SETQVKVWFQ NRRTKHKRMQQ EDEKG
EMX1	---------- ----R--R-- -K-H------- --Q--GS-S- ----▼------- ------Y-- QKL -E- GP
Emx1	---------- ----R--R-- -K-H------- --Q--GS-S- ----▼------- ------Y-- QKL -E-GP
Xemx1	---------- ----R--R-- -K-H------- --Q--SS-S- ----▼------- ------Y-- QKL -E-GP
EMX2	---------- ----R--- -- -K-H------- --Q--GS-S- T---▼------- ------F-- QKL -E-GP
Emx2	---------- ----R--- -- -K-H------- --Q--GS-S- T---▼------- ------F-- QKL -E-GP
Xemx2	---------- ----R--- -- -K-H------- --Q--GT-S- T---▼------- ------F-- QKL -E-GP

C

Otd	YPGVNTRK QRRERTTFTRAQLDVLEALFGKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQLQ
Otx1	--A▼TP-- --------S----------A------ ----- ----------▼ ----------Q -
Otx2	--A▼TP-- ----------A------ ----- ----------▼ ----------Q -

Figure 1.9

Structure and organization of Emx and Otx genes. A, Cloned cDNA and genomic organization of EMX1, EMX2, OTX1 and OTX2. B, Comparison of EMX1, EMX2 (human), Emx1, Emx2 (mouse), Xemx1, Xemx2 (*Xenopus*) homeodomains with *ems* homeodomain. C, Comparison of Otx1 and Otx2 (mouse) homeodomains with *otd* homeodomain. Dashes both in B and C indicate amino acid identity with *ems* and *otd* respectively, whereas arrowheads point to splice sites.

1.6 Otx AND Emx GENE EXPRESSION DURING MOUSE EMBRYO DEVELOPMENT

The onset of expression of the two Otx and the two Emx genes during mouse development varies. Otx2 is already expressed at the gastrula stage, in E5.5 mouse embryos (Simeone et al., 1993), whereas Otx1 and Emx2 can be detected in E8-8.5 embryos. Finally, Emx1 expression starts from E9.5 (Frantz et al., 1994; Gulisano et al., 1996). In fore- and mid-brain all four genes are expressed between day 9.5 and 10.5 of mouse development, in extended regions of the developing rostral brain, including the presumptive cerebral cortex and olfactory bulbs (Simeone et al., 1992). The Emx genes are also expressed in the uro-genital apparatus from the very beginning of its development (reviewed in Miyamoto et al, 1997; Cecchi et al, 2000).

1.6.1 Otx gene expression in early and midgestation mouse development

The Otx2 gene is the first of the four genes to be activated during mouse development. In fact, it is already expressed at about E5 in the entire epiblast, from which all embryonic tissues will originate. The same expression pattern is seen until E6.5. Between E7 and E7.5, Otx 2 expression domain progressively recedes to anterior regions, where it remains confined, contributing to the specification of the rostral brain (Simeone et al., 1993). Since E9, Otx2 appears to demarcate with a sharp boundary the division between mesencephalon and metencephalon; the gene will remain expressed in these region until late in gestation.

The progressive confinement of Otx2 expression from the entire epiblast to presumptive fore- and mid- brain neuroectoderm occurs concomitantly with progressive regionalization of cell fate within the epiblast. This Otx2 progressive confinement is certainly correlated with the expression of other developmental genes. For example, it is know that the expression pattern of early Hox genes (McGinnis and Krumlauf, 1992), in particular Hox2.9 (Wilkinson et al., 1989), undergoes to a progressive displacement towards anterior of the anterior border.

The role of Otx2 in gastrulation has also been analysed in different systems such as Xenopus (Pannese et al, 1995), chick (Bally-Cuif et al, 1994) and zebrafish (Mercier et al., 1995).

The localization of the OTX2 protein in mouse embryo parallels that of the corresponding transcript. An interesting feature is the gradient observed in the distribution of the protein, with a maximum in the anterior neuroectoderm and a gradually decreased level of expression in the more posterior ectoderm (Mallamaci et al., 1996).

Otx1 is first expressed in a large region of the anterior neural tube of 8.25-8.5 embryos (Simeone et al., 1992). Anterior-posterior delimitation of the Otx1 expression in the rostral neural tube is defined between E9 and E9.5 embryos. Dorsally, its expression domain is comprised of a continuous region including part of the telencephalon, diencephalon and mesencephalon. Ventrally, the Otx1 expression domain includes contiguous region of both diencephalon and mesencephalon with sharp anterior and posterior boundaries.

Considering that Otx2 is expressed in all epiblast very early, at E9.5 Otx2 expression domain contains that of Otx1 and includes the entire forebrain. Dorsally, it includes the entire telencephalon, the diencephalon and the mesencephalon. The anterior portion of this domain includes lamina terminalis and the presumptive basimedial striatum, whereas the posterior boundary coincides with that of the mesencephalon. Ventrally, the Otx2 expression domain includes contiguous regions of diencephalon and mesencephalon with an anterior boundary just posterior to the optic chiasma.

The two Otx genes are expressed in specific restricted regions of the developing brain in mouse embryos at mid-gestation (Simeone et al., 1993). They are expressed in the dorsal and basal telencephalon, diencephalon and mesencephalon, but not in the spinal cord. Their expression domains in mesencephalon show a sharp posterior boundary, both dorsally and ventrally at the level of the rhombic isthmus, already shown in earlier stages. From E9.5 onward, the expression of both genes clearly marks the posterior boundary of mesencephalon to the exclusion of presumptive anterior cerebellar domain. Ventrally, however, Otx1 expression re-appears posteriorly, in the anterior metencephalon, after a gap just posterior to the IV cranial nerve.

In the telencephalon, Otx1 expression is detectable in the presumptive cerebral cortex from its anterior boundary to its posterior limit. The hybridization signal is remarkably uniform across the cortex, without major variation. Sagittal sections reveal expression in the olfactory bulbs. Frontal sections show that the Otx1 domain includes

neopallium, hippocampal and parahippocampal archipallium and selected paleopallial and septal localisations (Simeone et al., 1992). Otx1 expression is also detectable in some noncortical basal telencephalic regions, namely in the germinal layer of the most lateral portion of lateral ganglionic eminence and in part of superior basimedial region.

Otx1 is also expressed in regions of the diencephalon: epithalamus, dorsal thalamus and mammillary region of posterior hypothalamus. Its expression domain does not include the ventral thalamus. A two-layered narrow stripe of expression is detectable at the boundary between the dorsal and the ventral thalamus, i.e. the zona limitans intrathalamica, which is the precursor of the lamina medullaris externa and mammillo-thalamic tract. Other localisation are the fasciculus retroflexus, the precursors of the habenulo-interpeduncular tract, stria medullaris, including the region surrounding the posterior commissure, the primordium of mammillo-tegmental tract, the epiphysis, the fornix and the sulcus lateralis hypothalami posterioris. Posterior to the diencephalon, Otx1 is expressed in mesencephalic regions of tectum and tegmentum, possibly at the level of presumptive dorsal periventricular bundle. Non-cerebral Otx1 localisations are in auricular and ocular regions, nasal cavities including external ductus and pharynx (Simeone et al., 1993). Later in development, Otx1 is expressed in deeper layers of telencephalic cortex. When the cortex is already in layers (i.e. P9-P16) Otx1 is expressed in a subpopulation of neurons in layer 5 and overall layer 6 except in the frontal area (Frantz et al., 1994).

Between E10.75 and E11.5, Otx2 expression disappears from the telencephalic cortex. In E12.5 embryos, Otx2 is no longer expressed in cortical telencephalon but only in a subset of presumptive non-cortical basal ganglia, in a complementary domain to that of Otx1. It is not expressed in anterior septal region but in septal regions contiguous to diencephalon and in germinal layer of anterior basimedial regions. Its expression in lamina terminalis confirms its very anterior expression shown earlier in development. Otx2 is expressed in areas of the diencephalon and mesencephalon with a pattern very similar to that of Otx1. Otx2 is also expressed in the anlage of neurohypophysis and in choroid plexuses.

Otx genes show an interesting pattern in developing cerebellum during early postnatal mouse life. A code of Otx gene expression seems to identify three distinct population of granule cell progenitors in the developing cerebellum between P5 and P16: a first population of granule cells progenitors expressing Otx1 only (lobules 1-5,

simple lobule); a second population expressing both Otx1, with a gradient from medium to low levels, and Otx2 with an opposite gradient from low to medium levels (lobule 6-9); finally, a third population with high levels of Otx2 expression (lobule 10 and flocculus) (Frantz et al., 1994). Otx genes show then opposite, overlapping A/P gradients during cerebellar development: Otx1 anterior to posterior, and Otx2 posterior to anterior, thus suggesting a specific role in establishing limits and identity during cell differentiation pathways in cerebellar cortex (Frantz et al., 1994).

Both genes are also expressed are expressed in the olfactory epithelium, as well as in the developing inner ear from early expression in the otic vesicles to epithelia in auricular ductus of sacculus and cochlea, and in developing eye (Simeone et al., 1993). Here the two genes are both expressed but with specific patterns maintained throughout eye development from their appearance in the optic stalk at E10. In E12.5 embryos Otx1 is expressed in the iris, in a peripheral region including ciliary bodies, sclera, external ectoderm, and the external sheath of the optic nerve, whereas in E17 embryos it is expressed in the pigmented epithelium of retina and in neurosensory retina.

1.6.2 Expression of Emx and Otx genes at E10

The developing neural tube in mouse embryos at E10 shows recognizable presumptive regions corresponding to the future anatomical subdivision. The entire neural tube consists of neuroepithelial cells in active proliferation and most of the specific differentiating events have not yet occurred.

In E10 mouse embryos, all four genes are expressed in contiguous regions contained within each other in the sequence Emx1<Emx2<Otx1<Otx2 (figure 1.10) (Simeone et al., 1992).

In the forebrain, Emx1 expression domain includes the dorsal telencephalon, with a posterior boundary slightly anterior to that between presumptive di-and telecenphalon.

Emx2 expression seems to define the boundary between the future dorsal and ventral thalamus. The gene is expressed in the dorsal and ventral neuroectoderm, its anterior boundary lying slightly anterior to that of Emx1 and the posterior one within the roof of the presumptive diencephalon.

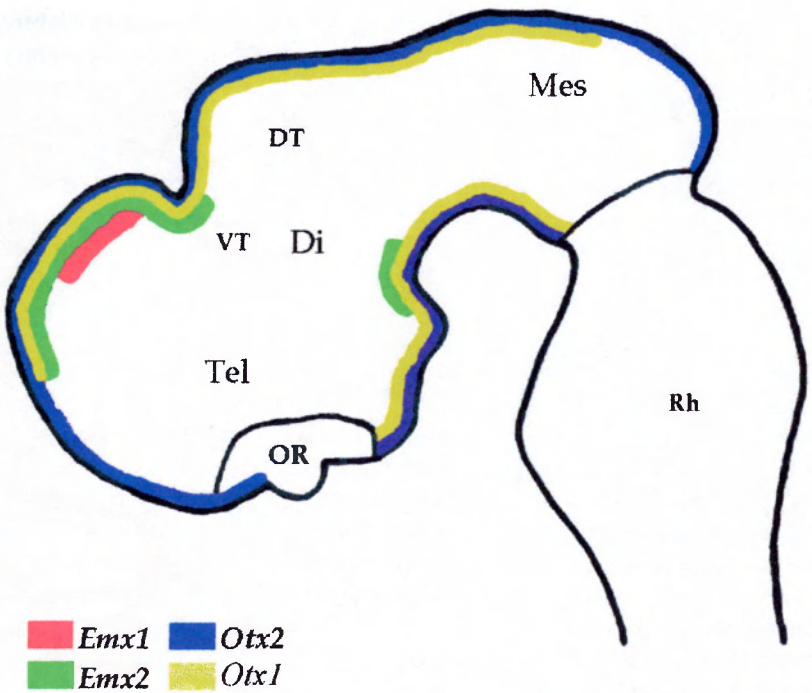


Figure 1.10

Summary of the expression domains of the four genes in the developing central nervous system of E10 mouse embryo. It is interesting to note that the expression domains of the four genes in the developing brain are nested. Moreover expression of Emx and Otx genes identifies a number of regions in forebrains. Dorsally, for example, the two Emx genes identify the presumptive cortical region. Tel, telencephalon; VT, ventral thalamus; Di, diencephalon; DT, dorsal thalamus; OR, optic recess; Mes, mesencephalon; Rh, rhomboencephalon.

Otx1 expression domain, that contains the Emx2 domain, includes dorsal and basal telencephalon, di-and mesencephalon (Simeone et al., 1993; Frantz et al, 1994; Gulisano et al., 1996), and its anterior boundary almost overlaps that of Emx2. Laterally the posterior boundary of Otx1 domain coincides with that of the mesencephalon.

Finally Otx2 expression domain contains the Otx1 domain both dorsally and ventrally; it includes the entire fore and midbrain, (Simeone et al., 1993). It is interestingly to note that an extremely anterior region, that includes the optic chiasma and the optic recess, is excluded from the Otx2 expression domain. This region, with the exception of the optic recess, is the site of expression of *Distal-less*-related (Dlx) genes (Price et al, 1991; Porteus et al.,1991; Robinson et al., 1991; Simeone et al, 1994;reviewed in Boncinelli et al., 1994). This anterior exclusion of Otx2 is in fact reminiscent of *otd* expression of the fruit fly embryo, which also retracts from the anterior pole. It suggests that, as in Drosophila, development of the extreme anterior terminus may be governed by a distinct genetic mechanism.

Otx1 and Otx2 expression is also detected in restricted regions of the diencephalon: epithalamus, dorsal thalamus and mammillary region of posterior hypothalamus, where the expression is almost exclusively restricted to cells of the ventricular zone. Otx expression is also found in mesencephalic regions of tectum and tegmentum, as well as in developing special sense organs, such as the olfactory epithelium, the developing inner ear and the developing eye, including the external sheath of the optic nerve (Simeone et al., 1993; Boncinelli et al, 1993).

Expression of Emx and Otx genes identifies several regions in forebrain (figure 1.10), some of which may correspond to presumptive anatomical subdivision. Dorsally, for example, it is clear that the two Emx genes identify a presumptive cortical region, part of which will give rise to neocortex and archicortex. Emx2 expression also defines the boundary between future dorsal (DT) and ventral thalamus (VT).

In summary, analysis of the mouse brain at E10 shows a nested pattern of expression of the four genes (figure 1.10) in brain regions defining an embryonic rostral brain opposed to hindbrain and spinal cord.

1.6.2 The Otx gene functions: area specification and axon fasciculation

Otx transcripts in di- and mesencephalon of E12.5-E14.5 embryos colocalize with boundary regions and presumptive axon tracts, including anterior and posterior commissures (Boncinelli et al, 1994). Expression is detected in precursor cells surrounding these structures, which might represent a border for pioneer axon tracts. This is particularly visible in posterior commissure and along the zona limitans intrathalamica (Boncinelli et al., 1993). Both Otx genes are also expressed around the developing optic nerve. This localization, like along the zona limitans intrathalamica, might be responsible for axon pathfinding and patterning. In this light, Otx genes are likely to provide a primary scaffold for specific axon pathways in the neuroepithelium of the developing forebrain.

It is therefore conceivable to think that Otx genes play at least two roles in head development, at two different stages. They first define territories or areas in rostral brain of E8-E10 mouse embryos, and provide later on a set of positional cues required for growing axons to follow pathways within the embryonic CNS. It is not clear whether the two functions are related. To this regard, it is also of interest to consider that in otd mutant flies, pioneer axons of the posterior commissures fail to develop normally as if appropriate positional cues were missing (Tessier-Lavigne, 1992).

Otx2 knockout embryos are early embryonic lethal, as they fail to gastrulate and stop developing at early midgestation (Matsuo eta., 1995; Acampora et al., 1995; Ang et al., 1996). By E8.25 the rostral part of the neural tube, corresponding to mid- and forebrain, is absent. This phenotype is consistent with a multi-functional role for Otx2 in gastrulating and patterning of rostral brain in the mouse.

On the other hand, frog embryos deriving from zygotes where Otx2 has been overexpressed (Pannese et al., 1995) show severely reduced trunk and tail structures as well as an expansion of anterior head structures including pharynx and forebrain (Boncinelli and Mallamaci, 1995).

It has been also demonstrated (Morgan et al., 1999) that a protein of the calponin family is able to implement Otx2-mediated determination of head versus trunk cell identity in early frog embryos, through the control of cell migration movements, highly reduced in early head cells and very active in early trunk cells. Very recently, the caudal border of the expression of Otx2 has been analysed as to its function in positioning the

mid/hindbrain organizer (isthmic organizer). Two different experiments, shifting the Otx2 expression domain either more caudally (Broccoli et al., 1999) or rostrally (Millet et al., 1999), show that the caudal limit of Otx2 expression is sufficient for the correct positioning of the isthmic organizer.

As it has been reported above, Otx1 is expressed in the VZ, particularly in the deepest layers of telencephalic cortex since their birth. Otx1 knockout mice are viable and show alterations in brain development as well as spontaneous epileptic seizures (Acampora et al., 1996). Particularly affected in these mice are the dorsal telencephalic cortex, mesencephalon and cerebellum. One can therefore attribute to Otx1 a role in regional specification of the developing brain. Very recently, it has been shown that the Drosophila otd gene can rescue the phenotype in Otx1 mutant mice, supporting the idea of the evolutionary conservation of otd/Otx gene function in head development in flies and mice (Acampora et al., 1998).

1.6.4 Emx gene expression in the developing mouse embryo

It has been reviewed above that the onset of Emx gene expression during mouse development varies: Emx2 gene begins to be expressed from E8.5, whereas Emx1 from E9.5. In mouse embryos, Emx genes are predominantly expressed in extended regions of the developing rostral brain, including the presumptive cerebral cortex, olfactory bulbs and olfactory epithelium. Although Emx2 is expressed both in dorsal and ventral telencephalon and hypothalamus, Emx1 expression is restricted to the dorsal telencephalon. Both genes are then expressed in the presumptive cerebral cortex from E9.5, with Emx1 expression continuing until long after birth; this period corresponds to major events in cortical neurogenesis, differentiation and migration and to establishment of synaptic connection. Emx genes might therefore determine the identity of the developing cortex.

Emx1 is also expressed in branchial pouches, and in the apical ectodermal ridge of developing limbs, whereas, Emx2 expression is detectable in the inner ear and digit primordia, as well as in adult ovary and testis (figure 1.11). Both Emx1 and Emx2 are expressed in early primordia of the organs fated to give rise to the excretory and reproductive system (Pellegrini, M. et al., 1997) (figure 1.11). Despite of the significant

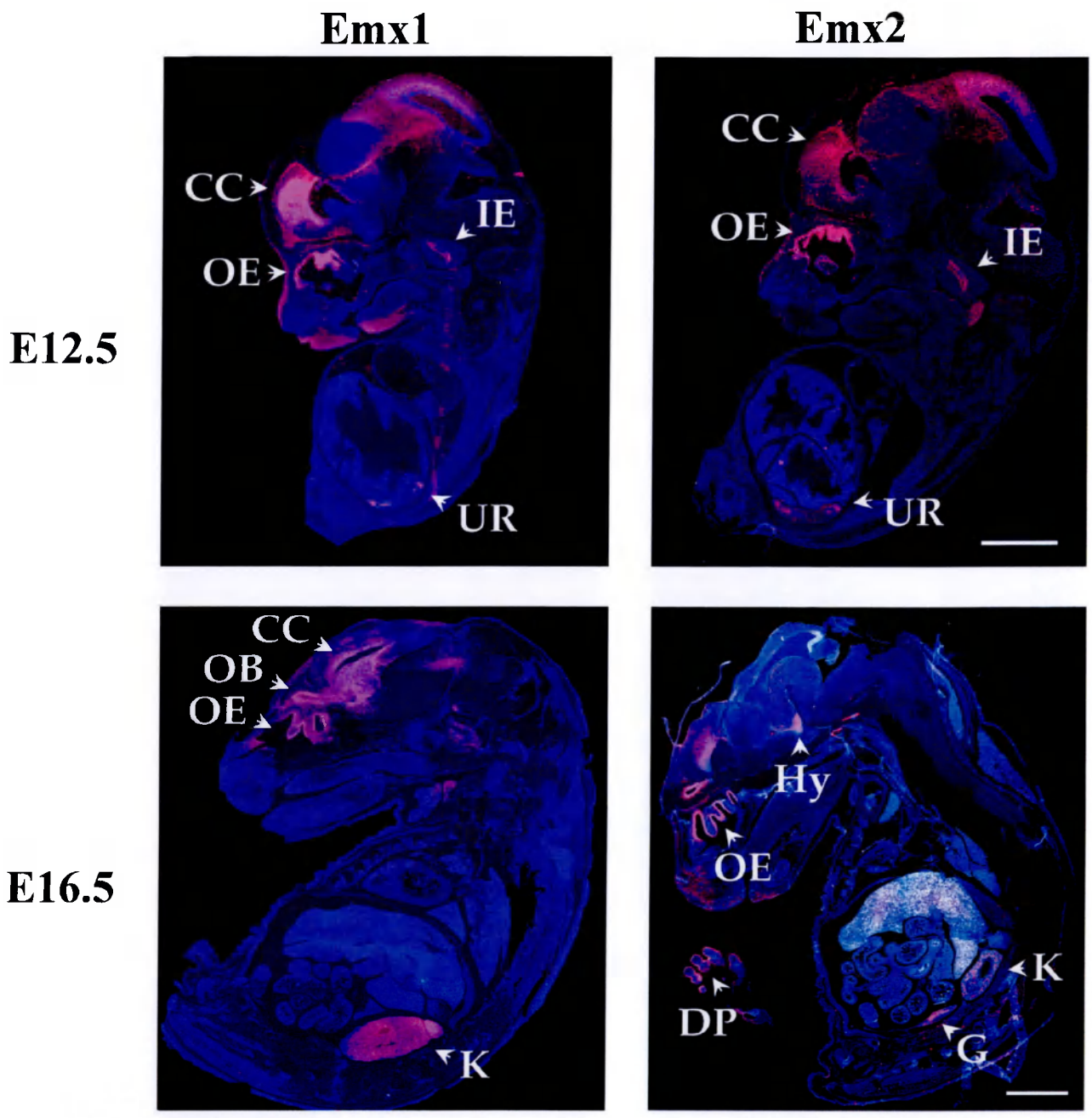


Figure 1.11

Comparison between the distribution of Emx1 and Emx2 mRNAs in E12.5 and E16.5 mouse embryos. Both genes highly expressed in the developing telencephalon, particularly in the presumptive cerebral cortex (CC), olfactory bulbs (OB) and olfactory epithelium (OE), from the very beginning of their development. They are also expressed in the primordia of the uro-genital ridge (UR), kidney (K) and (gonads), the digits (DP) and the inner ear (IE). As shown in the bottom right picture, Emx2 is also expressed in the hypothalamus (Hy). Scale bar, 1mm in the E12.5 embryos, and 4mm in the E16.5 embryos.

overlaps in time and between Emx1 and Emx2 expression in the forming uro-genital apparatus, their role seems to be different. Newborn homozygous null Emx2 mice lack uro-genital apparatus, whereas Emx1 homozygous null mice do not.

This thesis focuses primarily on the role played by Emx genes, and in particular of the Emx1 gene in cortical development; in this respect, I will dedicate the following session of the thesis to a more extensive description of Emx gene expression patterns in the developing and postnatal cerebral cortex.

1.7 EMX GENES IN CORTICAL DEVELOPMENT

1.7.1 Molecular regulation of Emx genes expression in mouse

In the fly ems gene, initially three distinct regulatory regions had been characterised, important for the mouse homologue gene expression (Taylor, 1998). Subsequently, two enhancer regions have been identified in the 5' regulatory region of the ems fly gene(Hartmann et al., 2000). A distal region of 2.6 kb drives the expression of the gene in the most anterior portion of the forebrain, and a proximal region of 4.1 kb controls expression in the trunk and ventral nerve cord (VNC).

Interestingly, mouse Emx2 overexpression in fly ems mutants has been shown to be able to rescue the phenotype in the head, but not in the trunk and VNC. Moreover, the mouse transcription factor *Gli3* is the only identified direct upstream positive regulator of Emx1 and Emx2 genes, acting in the control of dorsal telencephalic development by binding to their regulatory sequences (Theil et al., 1999). When Gli3 is deleted, as in the mutant extra-toes^{j} mouse (Tole et al., 2000a), Emx gene expression is reduced: the Emx2 transcript is present at very low levels and Emx1 is absent. Therefore, the disruption of normal dorsal telencephalon development, as well as the ectopic ventralization of the dorsal telencephalon observed in the extra-toes^{j} mutants, is expected to resemble the phenotype of Emx2/Emx1 double mutant mice.

As to Emx2 downstream genes, the EMX2 protein is thought to regulate the transcription of a number of genes via its homeodomain, but still very little is known about its target genes and the pathways controlled either directly or indirectly. *Wingless* (*wg*) and *engrailed* (*en*) have been suggested as targets of the Drosophila ems gene (Royet and Finkelstein, 1995). In mouse, the only in vitro-identified downstream target

gene is the corresponding *Wnt-1* (Iler et al., 1995), one of the earliest signals associated with CNS patterning, required for the formation of the midbrain during embryonic development. Wnt-1 has been proposed as direct target whose transcription is repressed through negative control by Emx2.

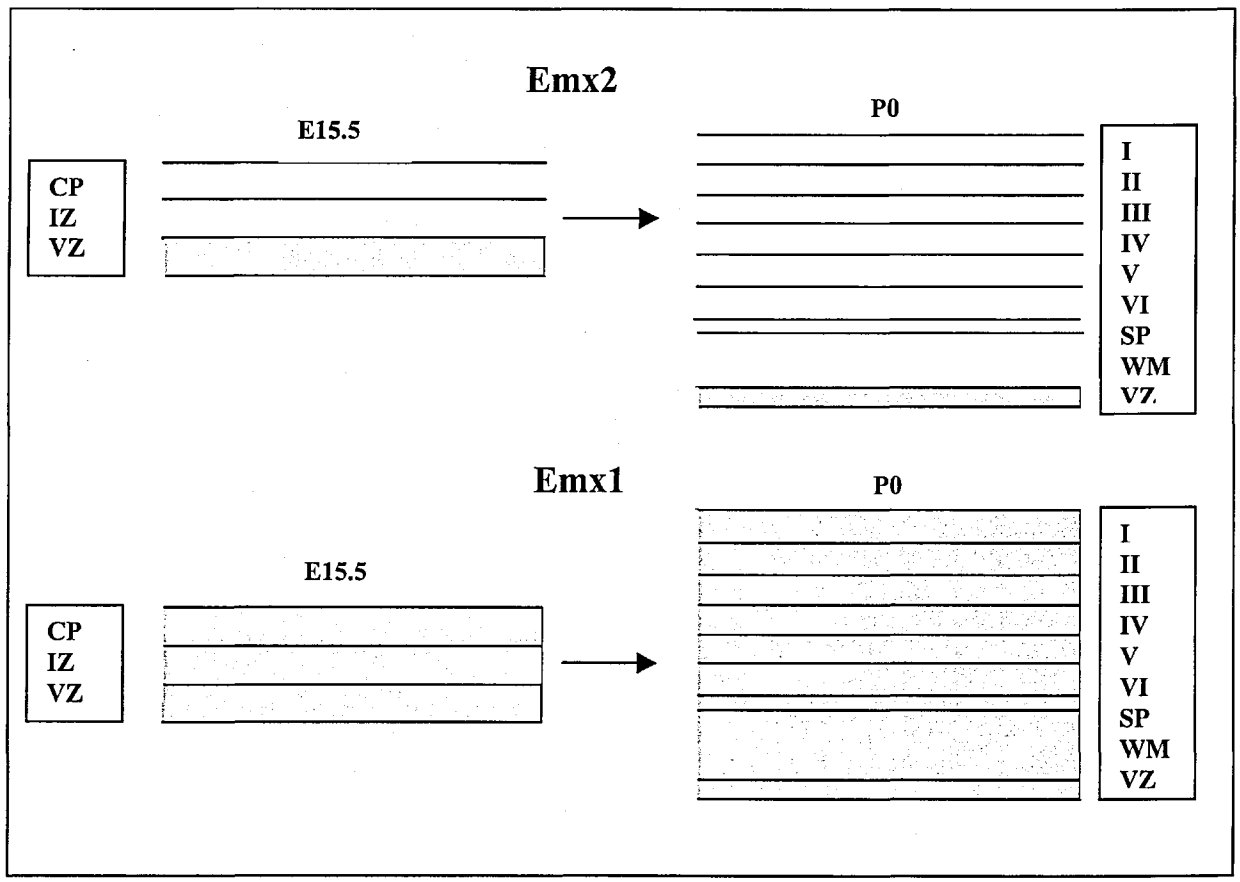
1.7.2 Emx2 gene expression in the developing forebrain.

Emx2 is one of the earliest dorsal markers of the developing cerebral cortex (reviewed in Cecchi and Boncinelli, 2000). The expression pattern of this gene has been extensively studied in mouse using in situ hybridization as well as immunohistochemistry techniques. Nevertheless, Emx2 expression has also been recently studied in early avian forebrain development, where it has been show to follow spatial and temporal expression patterns during a number of developmental phases (Bell et al., 2001).

It has been then showed that in mouse the gene is expressed at the three-somite stage in the latero-caudal forebrain primordia (Suda et al., 2001) and by E8.5 in anterior dorsal neuroectodermal regions of the embryo. By E9.5 the expression domain extends to the olfactory placodes, and is delimited by an anterior boundary, which overlaps that of Emx1, and by a posterior boundary, which is located within the roof of the presumptive diencephalon.

Particularly interesting is the expression of the gene in olfactory placodes, olfactory bulbs, olfactory epithelia of nasal chambers and in several cerebral locations related to olfaction. For example, Emx2 expression is detected in specific sites of hippocampal cortex, amygdala, specific areas of the basal cortex, hypothalamus, ventral and dorsal thalamus, habenulae, presumptive mammillary bodies, septal and tegmental regions. All these regions contain areas related to olfaction. It is also of interest to note that, in flies, ems is involved in the regulation of olfactory sense organs during development. Mutant ems flies lack primordia of antennal sense organs (Dalton et al., 1989), the main olfactory sensory structures of the Drosophila larva.

From E10.0 and during the formation of the cerebral cortex, the mRNA of Emx2 gene is detectable only in the neuroepithelium, whereas it is absent from most postmitotic neurons of the TF and CP, as confirmed by bromo-deoxyuridine (BrdU) pulse-labelling experiments (Gulisano et al., 1996) (figure 1.12). Moreover, at E12.5,



Fidure 1.12

Comparison of the expression domains of Emx1 and Emx2 in the cerebral cortex of the mouse. CP, cortical plate; IZ, intermediate zone; SP, subplate; VZ, ventricular zone; WM, white matter.

when the developing cerebral cortex practically consists only of the VZ, Emx2 expression at this level follows a gradient along the anterior-posterior axis; this gradient becomes more evident from E14.5 onward (Simeone et al, 1992; Gulisano et al., 1996). The hybridization signal appears to be stronger in the posterior dorsal telencephalon, and gradually decreases in intensity in anterior and ventrolateral regions. The anterior-posterior gradient of expression seems to follow the gradient in the maturation of neuroblasts during corticogenesis, the anterior regions having mature cells earlier than the posterior ones.

Recent data indicate that the distribution of EMX2 follows the same anterior-posterior and medial-lateral gradient. Interestingly, cortical neurogenesis follows the opposite gradient, with a rostro-lateral maximum and a caudo-medial minimum. EMX2 might therefore have a role either as an inhibitor of cell proliferation or as a positive regulator of cell differentiation. Graded expression of Emx2, both at the mRNA and protein levels, suggests that the gene contributes to cortical polarity, cell identity and patterning in an early arealization process that takes place in the VZ (O'Leary, D.D.M. et al., 1994). Moreover the localization of Emx2 in the proliferative layer of the developing cerebral cortex suggests a potential role for this protein in the control of neuronal proliferation and in the migration of cortical neuroblasts, as well as in the regulation of their subsequent differentiation.

By the end of gestation, Emx2 also marks specific cell layers in the hippocampus, amygdala and mammillary bodies, crucial structures involved in learning and memory processes. At P5, the number of cells expressing Emx2 in the cerebral cortex becomes irrelevant, as the thickness of the VZ is not significant compared with that of the TF and CP. However, high levels of expression are still detected in the hippocampus and, particularly, in the dentate gyrus (figure 1.14), suggesting a potential role for EMX2 not only in cell proliferation, but also in cognitive processes.

The analysis of the distribution of the EMX2 (figure 1.13) has revealed another cortical localization of the protein in the nuclei of the Cajal-Retzius cells (arrowheads in figure 1.13), the transient cell population that forms the most superficial layer of the developing cerebral cortex, the so-called marginal zone (MZ). As it has been already discussed in this thesis, the cells of the MZ are thought to be responsible for guiding radial migration of neurons, from the VZ to their final destination in the CP, through the

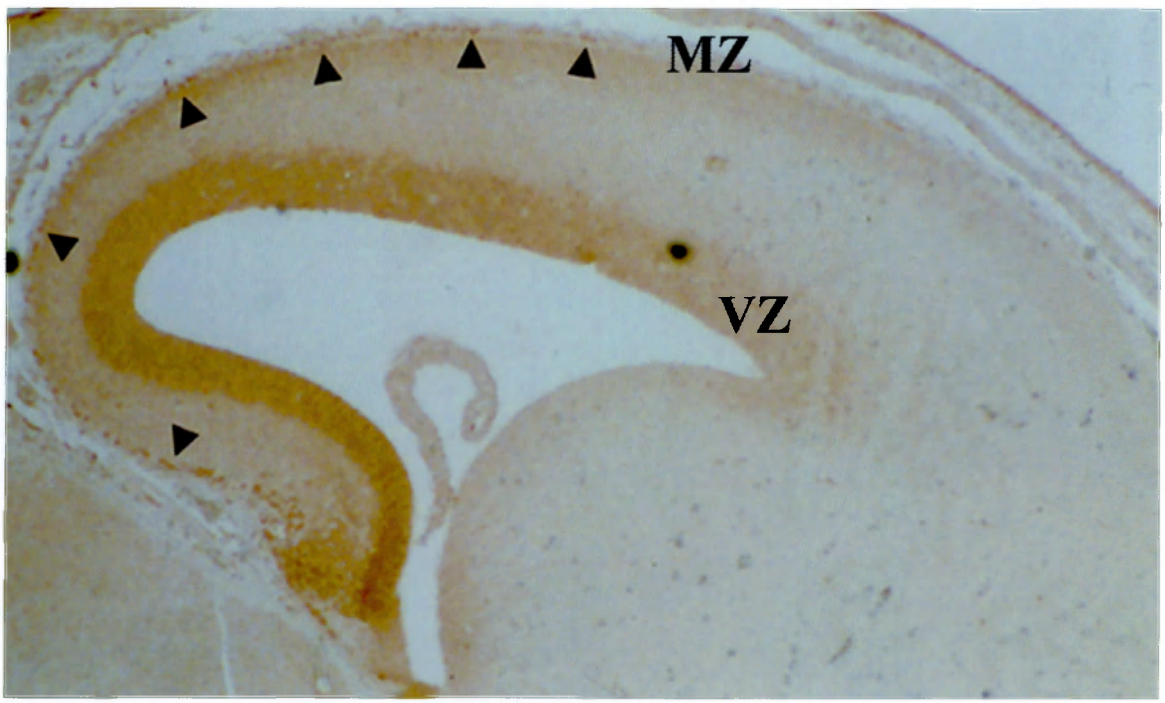


Figure 1.13

EMX2 protein distribution in E 15.5 mouse neocortex. The gene is expressed in the ventricular zone (VZ) of the forming cerebral cortex, and in the marginal zone cells (arrowheads). The medial-lateral gradient of expression is evident. Scale bar, 0.5 mm.

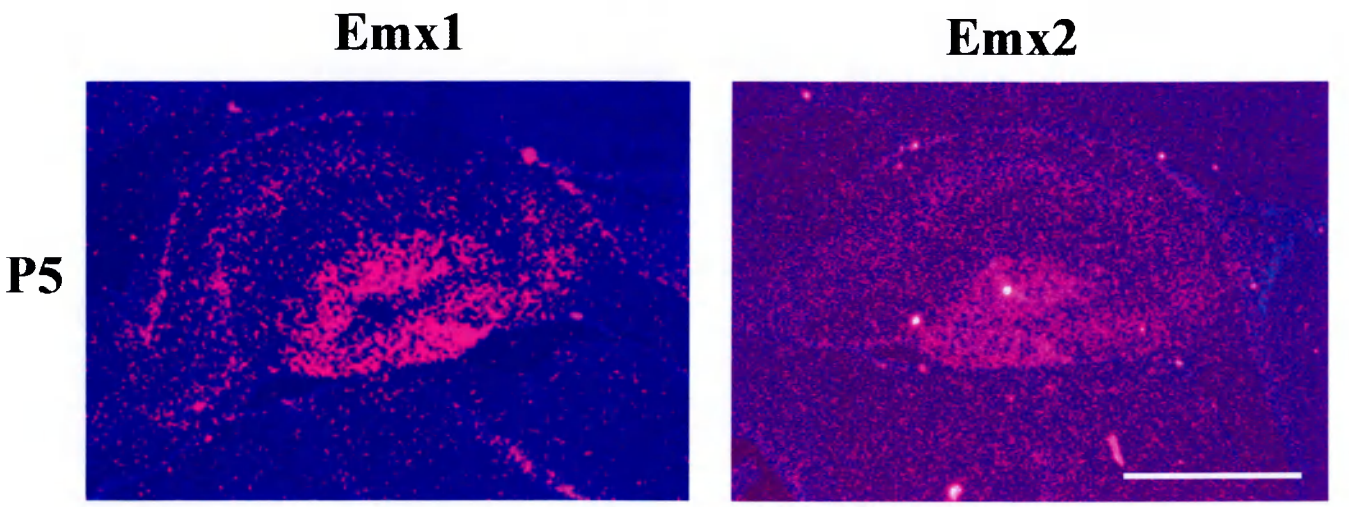


Figure 1.14

Emx1 and Emx2 expression in mouse hippocampus. Both mRNAs are detectable five days after birth in the hippocampal formation, and in particular in the dentate gyrus. The presence of Emx1 and Emx2 transcripts in these structures suggests important roles yet to be clarified. EMX2 has been shown to be involved in regulating the growth of the hippocampus. Scale bar, 0.6 mm.

secretion of an extracellular matrix protein named reelin (RELN) (Ogawa, M. et al, 1995; D'Arcangelo, G. et al., 1995; Hirotsune, S. et al, 1995; Alcántara et al., 1998; Curran and D'Arcangelo, 1998; Rice and Curran, 1999).

Taken together these data indicate that not only could Emx2 control the proliferation of cortical neuroblasts, but also it may regulate their subsequent migration process both directly and indirectly, as it is known that these cells finally settle in the cerebral cortex according to their birth date (McConnell, 1995).

Finally, it has been shown that ems in the fly embryo seems to be involved in the specification of trunk segments; similarly, the expression of Emx2 gene is also detectable in the dorsal-anterior base of the forelimb bud. These are proximal elements of the developing limb, and their formation is also controlled by Hox-cluster genes, including *Hoxc6*.

1.7.3 EMX2 functions in cortical development, as revealed by knockout mice and human schizencephaly patients

Analysis of Emx2 null mice has confirmed and specified the hypothetical functions of the gene in cortical development.

Emx2 gene has been knocked-out in mouse by homologous recombination in ES cells by two laboratories, obtaining a similar, striking mutant phenotype (Pellegrini et al., 1996; Yoshida et al., 1997). The Emx2 null mice die within a few hours after birth, because of the lack of the uro-genital apparatus (Miyamoto et al., 1997).

The brain of homozygous mutant embryos displays several abnormalities, which have been suggested to be related to the lack of both early and late expression of the gene (Yoshida et al., 1997). The development of the neocortical plate is impaired and olfactory bulbs are disorganized. In addition the olfactory epithelium fails to project to the olfactory bulbs (Yoshida et al., 1997). The dentate gyrus is missing; the hippocampus proper and the medial limbic cortex are greatly reduced in size (Pellegrini et a., 1996; Yoshida et al., 1997). EMX2 seems to contribute to the size of the hippocampus, rather than to the specification of the hippocampal field. The cells of the dentate gyrus are in fact identifiable with region-specific molecular markers, but they do not form a morphological gyrus (Grove, E.A. and Tole, S., 1999; Tole, S. et al., 2000).

Interestingly, a similar phenotype is seen in Lhx5 null embryos, where precursors cells of the hippocampal anlagen are specified, but fail to exit the cell cycle, resulting in the absence of the dentate gyrus (Zhao, Y. et al, 1999).

A detailed analysis of Emx2 null embryos (Mallamaci et al., 2000), has shown that these embryos lack Cajal-Retzius cells from the MZ. As a consequence, the settling of radial glia is impaired and neurons display a reeler-like migration pattern. This results, at the end of the prenatal period of neocortical development, in a reeler-like phenotype of the cortex, indicating an involvement of the gene in neuronal migration processes.

The intriguing hypothesis that Emx2 might play a role in cortical migration has led to the analysis of other phenotypes related to cortical dysgenesis, particularly to cell proliferation and migration disorders, such as the human schizencephaly, lissencephaly and double cortex syndromes.

Schizencephalies are rare human developmental defects of the cerebral cortex (Granata et al., 1996). These congenital brain malformations are characterized by full-thickness clefts within the cerebral hemispheres. Affected patients display different motor and mental problems, according to the severity and extent of the brain malformation. They are frequently affected by epilepsy. A genetic analysis of 18 schizencephalic patients has shown that 13 of them carry heterozygous mutations of the EMX2 gene (Faiella et al., 1997). For at least some cases a correlation can be made between the molecular defect and the severity of the disease. In fact, severe mutations (i.e. frameshift or splicing mutations) are associated with severe bilateral forms of schizencephaly, whereas milder mutations (i.e. missense mutations) are associated with lighter manifestations of the disorder. This finding supports the hypothesis that EMX2 is required for the correct formation of the human cerebral cortex.

More recently, it has been reported that Emx2, functioning in concert with other factors, and in particular Pax6, is involved in the specification of neocortical areas identity (Bishop, K. et al, 2000; Mallamaci, A. et al., 2000).

The process of cortical arealization is described in detail elsewhere in this thesis. Nevertheless, it is important to remind here, that the specification of neocortical areas is controlled by an interplay between extrinsic and intrinsic mechanisms. The major extrinsic influences depend on the axons coming from the thalamus, that reach and innervate the cortex.

Early phases of arealization, as reviewed above, occur before the arrival of thalamo-cortical projections and depend on intrinsic (genetic) cues.

Interestingly, it has been shown that arealization of the Emx2 mutant cortex is altered (Mallamaci et al, 2000; Muzio et al., 2002), with an expansion of rostral areas and a contraction of the caudal ones. An opposite effect on arealization is observed in Pax6 mutant neocortex (Bishop, K. Et al., 2000). Emx2 and Pax6 may act independently or in combination (possibly with other transcription factor genes) to specify neocortical areas. Areas in the neocortex have sharp borders; the graded expression patterns of Emx2 and Pax6 could be translated to regulate some downstream genes with restricted patterns related to specific neocortical areas.

Studies in Drosophila have shown that gradients of transcription factors can be translated into sharply bordered expression patterns of downstream genes (Rusch, J. And Levine, M., 1995; Stanojevic, S. et al., 1991; Small, S. et al., 1996).

Finally, it has been seen (Bishop, K. et al., 2000; Mallamaci, A. et al., 2000) that arealization defects of Emx2 mutant cortex could be due not only to the absence of EMX2 in the cortex but also to abnormalities in thalamo-cortical afferents and cortico-thalamic efferents. In other words, Emx2 might have a role in regulating axon guidance molecules that control thalamo-cortical and cortico-thalamic targeting.

1.7.4 Emx1 expression in the developing and postnatal cerebral cortex

Emx1 gene expression is exclusively confined to the dorsal telencephalic neuroepithelium since its first expression in E9.5 mouse embryo (Simeone et al, 1992a, b); the posterior boundary of Emx1 expression domain is slightly anterior to that between the presumptive di- and telencephalon (figure 1.10). In other words, Emx1 is exclusively expressed in the presumptive cerebral cortex, even before neurogenesis starts. By contrast, no expression is detected in the basal telencephalon. This finding has prompted scientists to hypothesize that Emx1 would play a role exclusively within the dorsal part of the forebrain.

Emx1 gene is expressed in most cortical neurons, either proliferating, migrating, differentiating or fully differentiated and organized in mature cerebral cortex (figure 1.12) (Gulisano et al., 1996). It has been shown that the Emx1 transcript (figure 1.11) is distributed across the cerebral cortex (VZ, IZ and CP), even if the signal is present at

different intensities depending on developmental stage. Emx1 is also highly expressed in subplate neurons. The majority of subplate neurons disappear in adult life, and in mouse over 80% of the cells die by P21 (Allendoerfer and Shatz, 1994); these neurons are thought to take part in early functional circuitry, both receiving synaptic inputs from thalamic afferents and making axonal projection to the CP (Allendoerfer and Shatz, 1994), and cooperating in the formation of the corticofugal projection system. They may function as a cellular scaffold to drive correctly incoming thalamic axons to cortical targets and in turn cortical efferents to the thalamus.

This observation suggests a possible involvement of Emx1 in some of these crucial events as well as in defining specific cellular identities in the cerebral cortex.

As with Emx2, Emx1 transcript is largely present in the hippocampus and dentate gyrus of postnatal and adult mice (figure 1.14). The hippocampus has been shown to be a source of stem cells and new neurons (Johansson et al., 1999). Moreover cell proliferation experiments performed in the neocortex of adult primates (Gould et al., 1999) indicate that new cells differentiating into neurons originate in the SVZ. They then migrate to neocortical regions where they mediate behavioral plasticity and are involved in learning and memory processes. Neuronal proliferation in the subgranular zone of the dentate gyrus has also been observed in the adult human hippocampus. It will be of interest to study the role of Emx genes in this context, in particular if their functions are restricted to cell proliferation or involved in establishment neuronal connections.

Interestingly, analysis of the EMX1 protein distribution, which was expected to overlap that of the corresponding mRNA, revealed more new localizations (Briata et al., 1996). Most of the EMX1 signal can be detected in the nuclei of cells of the developing telencephalon, including the presumptive cerebral cortex, olfactory bulbs and hippocampus. Just like the transcript, the protein is present in virtually all cortical neurons during proliferation, migration differentiation and maturation. EMX1 is first detectable in the dorsal telencephalon at E9.5, as the corresponding mRNA, where it persists until E11.5. The signal is particularly strong in the VZ between E10.5 and E17.5. At birth and shortly after, it becomes more intense in the forming layer V and VI.

Briata et al., 1996 have also shown EMX1 protein along the entire length of the developing and postnatal olfactory nerve. According to these authors, the protein could

be detected from the olfactory epithelium of the developing nose to the terminals touching the olfactory bulb. This localization makes it very difficult to define a role for a transcription factor along axons out of the nucleus. It is though conceivable that EMX1 may assist olfactory sensory neurons in finding their way to specific glomeruli.

Recent data, coming from experiments performed in rats at different stages of postnatal life and in adult animals, have shown that Emx1 expression in the cerebral cortex is restricted to pyramidal neurons (Chan et al., 2001). Pyramidal neurons constitute the major projection neurons of the neocortex; they are the projection neurons of the cortex, found in all layers except layer I (Lorente de Nó, 1949) and utilize the amino acid L-glutamate as neurotransmitter (Parnavelas et al, 1989). These neurons are originated in the ventricular zone that lines the telencephalic ventricles and migrate to their position in the cortex guided by radial glia (Mione et al., 1997; Tan et al., 1998). By contrast, the cortical non-pyramidal cells contain the neurotransmitter GABA and derive from the ganglionic eminence of the ventral telencephalon (Anderson et al., 1997; Lavdas et al., 1999; Parnavelas, 2000).

Interestingly, it has been observed that the vast majority of Emx1-expressing cells in the cortex also contain glutamate, which is a marker of pyramidal neurons (Chan et al., 2001). This finding implies that Emx1 can be used as a marker of pyramidal cell lineage. Moreover Emx1 gene expression starts in the dorsal forebrain even before the generation of the first cortical neurons; this may suggest that this homeobox-containing gene is essential for the pyramidal phenotype.

It is very hard to ascribe a precise function to EMX1 in the developing telencephalon. However, its presence throughout corticogenesis might confer cellular identity to cortical neurons, and in particular to pyramidal neurons.

Homeobox genes have often been associated with an instructive role in conferring positional identity (reviewed in Krumlauf, 1994); some of these genes, i.e. Dlx1 (Price et al., 1992) and Dlx2 (Bulfone et al., 1993), Dbx (Lu et al., 1992), Nkx-2 (Price et al., 1991) seem to be specific for the specification of basal forebrain regions.

Conversely, Emx1 gene is expressed in a restricted domain coincident with the dorsal telecephalon, that is endowed with specific local cues that are able to drive a transplanted basal cell toward a specific cortical fate (Fishell, 1995). It has therefore hypothesized a role of Emx1 in originating and/or maintaining local identity cues specific for dorsal telencephalon.

As it has been revealed by the analysis of Emx2 null embryos, genetic manipulation of these homeobox-containing gene represent a powerful tool for the study of brain development, in particular, of cortical development. Any alteration that occurs in this process is thought to be responsible for severe developmental defects and pathologies of the nervous system (i.e. schizencephaly, as described above).

In order to uncover the functions of Emx1, mice lacking a functional Emx1 gene have been produced (Qiu et al., 1996; Yoshida et al., 1997).

1.7.5 Emx1 null mutant mouse

The constitutive inactivation of Emx1 has been obtained by two laboratories, and phenotypes observed in the mutant embryos partially overlap.

In 1996, Qiu et al. generated a mouse strain with a mutation of Emx1 that results in a 2.2 Kb deletion, including exon 1 and part of intron 1 of the gene; the mutation results in removal of the N-terminus of the EMX1 protein. After homologous recombination in ES cells, they were able first to isolate positive clones and generate chimeric mice; subsequently, they obtained homozygous mice for the mutant allele, on a 129sv/J genetic background.

The analysis of the mutant phenotype has revealed that these mice are viable and fertile and do not exhibit any obvious morphological or behavioural defects, with the exception that they lack most or all of the corpus callosum.

129/J mouse strain is one of the four major inbred strains known to suffer from agenesis of the corpus callosum. In these mice, absence of the corpus callosum can be found in about 20-30% of adults (Ozaki and Wahlston, 1993); in other words, defects of the corpus callosum occurs spontaneously with incomplete penetrance and are attributed to recessive multifactorial inheritance It might, therefore, be possible that the alteration of the callosal tract in Emx1 mutants is strongly influenced by the genetic background, rather than being exclusively determined by the Emx1 gene deletion. To support this idea, a recent study shows that mice with a C57BL/6 background, homozygous for a mutation in the β-amyloid precursor protein (βAPP) gene, have a normal corpus callosum; on the contrary, only 1.5% of the βAPP homozygous mutant mice with 129/Sv background show a normal corpus callosum (Magara et al., 1999), indicating

that the genetic background can change the pattern of corpus callosum defects in βAPP homozygous mutant mice.

The histological appearance of the neocortex, limbic cortex (hippocampus, entorhinal, cingulate, insular) and paleocortex (olfactory bulb and piriform cortex) in the mutant mice was indistinguishable from wild-type littermates. Molecular properties of the mutant cerebral cortex were analysed by immunocytochemistry and in situ hybridisation using molecular markers such as the glutamate GlnR2-3 receptor or Tbr1 and Id2, without showing any abnormality.

In 1997, Yoshida et al. introduced a mutation in Emx1 gene by homologous recombination in ES cells obtaining a partial deletion of the coding region. Emx1 null mice were generated in C57BL/6-CBA mouse strain. These authors reported that the frequency of $Emx1^{-/-}$ mice matched that expected based on Mendelian ratios and that the mice develop and become adults. The mutant embryonic brains are indistinguishable from the wild-type ones, whereas the adult brain shows subtle defects that are restricted to the forebrain. Disorganized fasciculation in the corpus callosum is evident in a significant portion of Emx1 null mice. The CP and the white matter are thinner than in wild type animals, and the subplate is hardly visible. The hippocampus and dentate gyrus are sometimes smaller but always present.

These researchers cannot detect abnormalities in the developing brain of Emx1 null embryos, but they can find subtle defects in the adult; this fact may be attributable to the later expression of the gene and then suggest that the gene has some roles in driving late phases of neurogenesis. The defects of the corpus callosum reported by these authors seem to confirm the previous data from Qiu et al. (1996) and to suggest a possible involvement of Emx1 in the regulation of axon guidance during development.

Nevertheless, it has been more recently demonstrated that corpus callosum deficits in Emx1 mutant mice are strain-dependent (Guo H. et al, 2000; our unpublished data). Indeed, 100% of Emx1 mutant mice with C57BL/6 genetic background have a normal corpus callosum, indicating that Emx1 gene does not contribute directly to acallosal or dyscallosal phenotype associated with Emx1 mutant mice.

1.8 AIM OF THE THESIS

Little is known about the molecular mechanisms underlying the complex changes that occur during the development of the forebrain, and about the genes that are involved in this process.

In the past decade, several families of genes coding for transcription factors, including homeobox genes, have been cloned in Drosophila (see above for details) and their homologues have been shown to have overlapping patterns in the developing brains of rodents (Rubenstein and Puelles, 1994; Wilson and Rubenstein, 2000). Several of these gene including Emx1 and Emx2 as well as Otx1 and Otx2, are expressed in the cerebral cortex (see above).

Since the study of Otx and Emx genes began, their expression profiles have suggested the involvement of these genes in directing brain development. Different approaches have been chosen to investigate their functions; in particular the generation of knockout mice for each of the four genes provided insights into their functional roles.

When I started my Ph.D three years and a half ago, there were already interesting and promising data coming from the analysis of Emx2 mutant mice, suggesting the involvement of this gene in proliferation, migration and differentiation of cortical cells as well as in specification of cortical areas. In fact, its multiple functions make Emx2 gene instrumental at all stages of cortical development. On the contrary, our knowledge of the function of Emx1 gene in the formation and maturation of the cerebral cortex was still very limited. Therefore, the aim of my Ph.D. research project was to uncover the functions that Emx1 gene might play in CNS development, and in particular, in cortical development.

Previous studies of Emx1 null mice had not shown any direct involvement of Emx1 gene neither in the early construction of the cerebral cortex nor in postnatal phases of cortical development and adult life. On the other hand, its expression profile makes Emx1 an excellent marker of cerebral cortex; very recently, Chan et al. (2001) have demonstrated that Emx1 gene is exclusively expressed by cortical pyramidal cells, that constitute the majority of cortical neurons.

Starting from those reports, I hypothesised that the loss of Emx1 function causes morphological defects or neurochemical changes that could not be detected by gross histological or histochemical methods. Another possibility was that Emx1 function

could be compensated for by other genes especially those having an overlapping expression pattern.

The fact that Emx1 is expressed in pyramidal neurons both in developing and mature cortex would suggest that this gene could be involved in the specification and maintenance of the pyramidal phenotype.

So, the aim of my work was to provide a detailed description of the changes that occur not only in the developing brain of Emx1 null embryos, but also in the neuronal composition, connectivity and synaptic connection in the cortex of $Emx1^{-/-}$ mice.

Information about the role of Emx1 together with data coming from the analysis of mutant mice for other genes involved in the development of the brain might increase our knowledge on this process. Considering the high degree of evolutionary conservation of these genes (see above for details), it is conceivable that all this information could be used to understand mechanisms of human brain development and function.

To this end, I started from the preliminary observation that, in the absence of a functional Emx1 gene, the cerebral cortex develops without major defects in mouse. Then, I decided to focus on the human EMX1 gene in order investigate if mutations, arisen spontaneously in this gene, could be related to functional or electrical disturbances of the cerebral cortex, more than development morphological or anatomical abnormalities. In particular, I chose to analyse genomic DNA samples from patients affected by different epileptic syndromes, both sporadic and congenital.

In the next paragraphs, I describe the cells that compose the CNS and the major groups of cortical neurons. Subsequently, I briefly introduce the epilepsy, and the classification and the causes, where known, of epileptic syndromes.

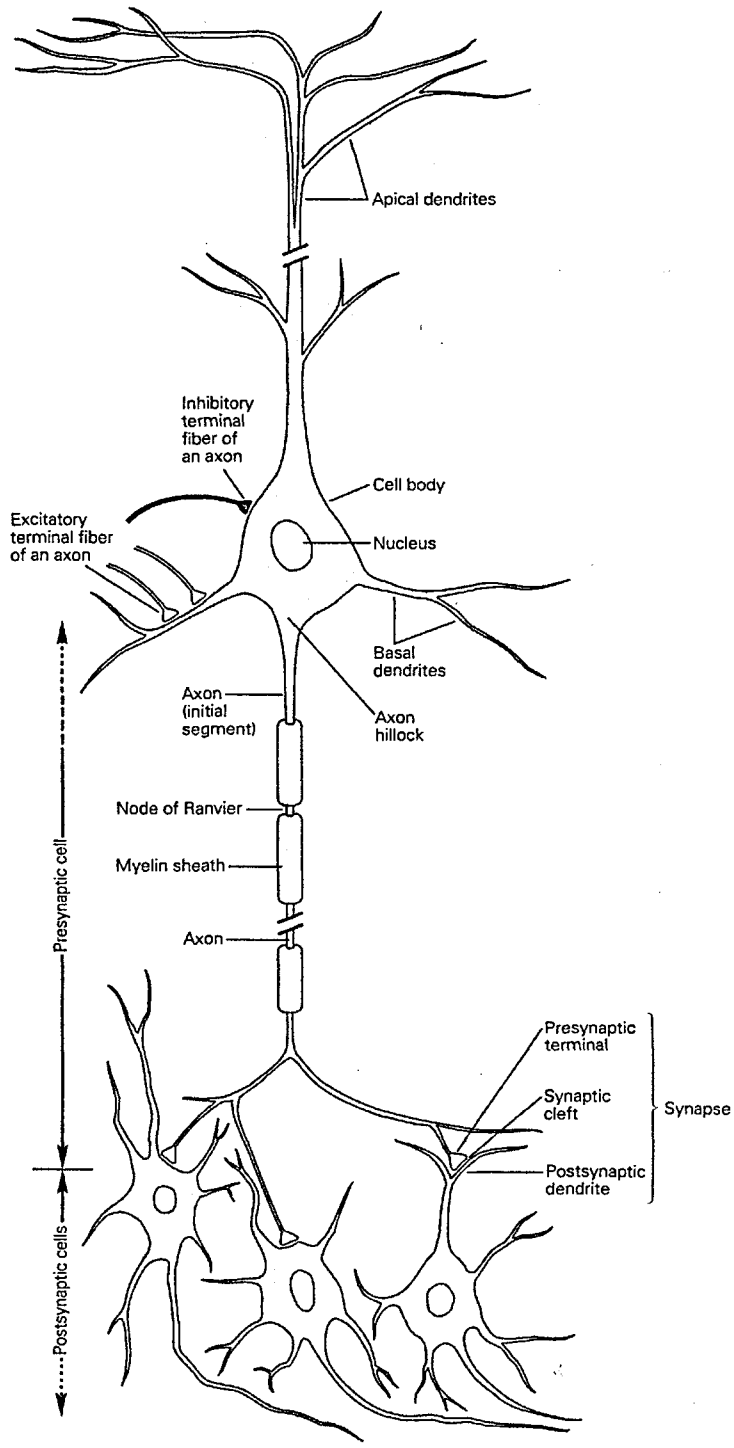


Figure 1.15

Structure of a neuron. Most neurons in the vertebrate CNS have several features in common. The cell body contains the nucleus, and gives rise to two types of cell processes: axons and dendrites. Axons can vary greatly in length, and many of them are insulated by a myelin sheath that is interrupted at regular intervals by the nodes of Ranvier. Branches of the axon of one neuron (the pre-synaptic neuron) transmits signals to another neuron (the post-synaptic cell) at a site called synapse.

1.9 CELLS OF THE NERVOUS SYSTEM

There are two main classes of cells in the nervous system: nerve cells (neurons) and glial cells. There also are other supporting cells like blood vessels, meninges, immune cells and fibroblasts.

NERVE CELL

A typical neuron has four morphologically defined regions: the cell body, dendrites, the axon, and presynaptic terminals (figure 1.15). The major characteristic of nerve cells is the ability to generate active electrical signals and each region has distinctive signalling functions.

The cell body is the metabolic center of the cell, and gives rise to two kinds of processes: several short *dendrites* and one long, tubular *axon* (figure 1.15). Dendrites branch out in tree-like fashion and are the main apparatus for receiving incoming signals from other nerve cells. In contrast, the axon extends away from the cell body and is the main conducting unit for carrying signals to other neurons (figure 1.15). An axon can convey electric signals for very long distances. These electric signals are called *action potentials*, and are rapid, transient and all-or-none nerve impulses. Action potential are initiated at a specialised trigger region at the origin of the axon, called *axon hillock*; from there they are conducted down to the axon without failure or distorsion. The amplitude of an action potential travelling down the axon remains constant because the action potential is an all-or-none impulse that is regenerated at regular intervals along the axon.

Action potentials constitute the signals by which the brain receives, analyses and conveys information. These signals are highly stereotyped throughout the nervous system, even though they are initiated by a great variety of events in the environment. In other words, the information conveyed by an action potential is not determined by the form of the signal but by the pathway of signal travelling in the brain.

The speed at which the action potentials are conducted, is highly increased by the presence of myelin that forms a fatty, insulating sheath on axons. This sheath is interrupted at regular intervals by the nodes of Ranvier (figure 1.15). It is at these uninsulated spots on the axonal membrane that the action potential becomes regenerated.

Near its end, the tubular axon divides into fine branches that form communication sites with other neurons. The point at which two neurons communicate is called *synapse* (figure 1.15); the nerve cells transmitting the signal is the *presynaptic cell*, whereas the cell receiving the signal is named the postsynaptic cell. The presynaptic cell transmits signal from the swollen ends of its axon's branches, called *presynaptic terminals*. However, the presynaptic cell does not touch the postsynaptic one, but the two cells are separated by a space, named *synaptic cleft*.

Neurons can be subdivided into at least three different groups on the basis of their shapes, specifically, the number and the form of the processes arising from the cell body. Those groups are: unipolar, bipolar, and multipolar (figure 1.16).

Unipolar are the simplest nerve cells; they have a single process, which usually gives rise to many branches. One branch serves as axon, the others as dendritic receiving structures. Unipolar cells are characteristic of invertebrate nervous system (figure 1.16).

Bipolar neurons have an oval-shaped soma that gives rise to two processes: a dendrite, that conveys information from the periphery of the body, and an axon that carries information toward the CNS. Many sensory cells are bipolar, including those in the retina of the eye (figure 1.16) and in the olfactory epithelium of the nose.

Multipolar neurons predominate in the nervous system of the vertebrate. They have a single axon and typically many dendrites emerging from various points around the cell body. Multipolar cells vary greatly in shape, especially in the length of the axon and in the number, length and intricacy of dendritic branching. The large diversity of these cells is illustrated in figure 1.16 with three examples.

Neurons are also commonly classified into three major functional groups: sensory, motor and interneuronal. *Sensory neurons* are responsible of the perception and carry information from the body's periphery into the nervous system. *Motor neurons* carry command from the brain or the spinal cord to the muscles or glands. *Interneurons* constitute the largest class, consisting of all nerve cells that are not specifically sensory or motor neurons.

Finally, it is to note that the shape of a neuron as well as information about its position, origin and destination in the neuronal network, supplies valuable clues to its function.

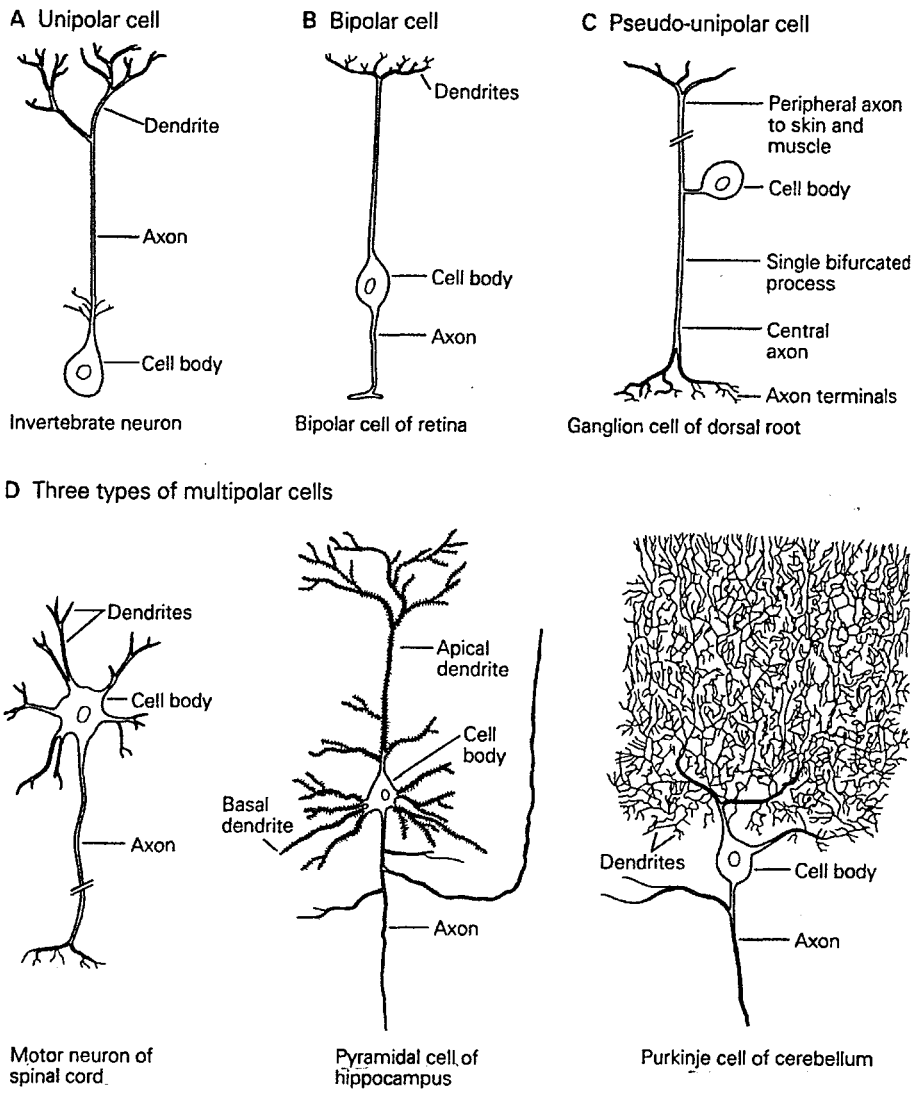


Figure 1.16

Schematic drawings of the different shapes of neurons. Neurons can be classified as unipolar (A), bipolar (B), and multipolar (D) according to the number of processes that originate from the cell body (details in the text). Certain neurons that carry sensory information belong to a subclass of bipolar cells designated as pseudo-unipolar. As such cells develop, the two processes of the embryonic bipolar cell become fused and emerge from the cell body as a single process. This outgrowth then splits into two processes, both of which function as axons.

GLIAL CELLS

The glial cells are between 10 and 50 times more than neurons in the CNS of vertebrate. These cells have multiple vital functions: they support neurons; produce myelin to insulate nerve cell axons; some of them are scavengers, removing debris after injury or neuronal cell death; other perform housekeeping chores that promote efficient signalling between neurons; during development, guide migration of neurons and direct the outgrowth of axons; a class of glial cells form an impermeable lining in the brain's capillaries and venules (the blood-brain barrier) that prevent toxic substances from entering the brain.

Glial cells in the vertebrate nervous system are divided in two major classes: *microglia* and *macroglia*. Microglia are phagocytes that are mobilized after injury, infection, or disease. They arise from macrophages outside the nervous system and are physiologically and embryologically unrelated to the other cell types of the nervous system.

Macroglia consists of three types of glial cells: oligodendrocytes, Schwann cells, and astrocytes. Oligodendrocytes and Schwann cells are small cells with relatively few processes. Both types are responsible of the formation of the myelin sheath that insulates axons by tightly winding their membranous processes around the axon in a spiral. Oligodendrocytes are found in the CNS and are able to envelop an average of 15 axonal internodes each. By contrast, Schwann cells, which occur in the PNS, each envelop just one internode of only one axon. The types of myelin produced by oligodendrocytes and Schwann cells differ to some degree in chemical makeup.

Astrocytes are the most numerous of glial cells, characterized by irregular, roughly star-shaped cell bodies. They have long processes, some of which terminate in end-feet. Some astrocytes form end-feet on nerve cells in the brain and spinal cord that may play a role in bringing nutrients to these cells; other are involved in the formation of the blood-brain barrier and many other functions.

There is no evidence that glial cells are directly involved in electrical signalling. Signalling is the function of neurons.

1.10 NEURONAL CELL TYPES AND NEUROTRANSMISSION PROPERTIES OF THE MAMMALIAN CEREBRAL CORTEX

LAYERS OF THE NEOCORTEX

The mammalian cerebral cortex is organized in six layers, numbered from the outer surface (pia mater) of the cortex to the white matter.

Layer I is an acellular layer called *molecular layer*. It is occupied by dendrites located deeper in the cortex and axons that travel through or form connections in this layer.

Layer II is comprised mainly of small spherical cells called granule cells and therefore is called the *external granule cell layer*.

Layer III contains a variety of cell types, many of which are pyramidally shaped; the neurons located deeper in layer III are typically larger than those located more superficially. Layer III is called *external pyramidal cell layer*.

Layer IV is made up primarily of granule cells and is *called internal granule cell layer*.

Layer V, the *internal pyramidal cell layer*, contains mainly pyramidally shaped cells that are typically larger than those in layer III.

Layer VI is a fairly heterogeneous layer of neurons and is thus called polymorphic or multiform layer. It blends into the white matter that forms the deep limit of the cortex and carries axons to and from the cortex.

Each layer also contains additional elements: thus, layers I-III contain the apical dendrites of neurons that have their cell bodies in layers V and VI, while layers V and VI contain the basal dendrites of neurons with cell bodies in layers III and IV.

It is also to note that not all cortical regions have the same laminar organization, but for example in humans the region of the occipital cortex that functions as the primary visual cortex has extremely prominent layer IV while the primary motor cortex has essentially no layer IV.

CORTICAL CELL TYPES AND NEUROTRANSMISSION PROPERTIES

The neocortex contains millions of neurons that show a variety of morphologies and chemical characteristics. These neurons can be subdivided into projection (or pyramidal) and local circuit nerve cells (interneurons), regarding their axonal extent (reviewed in DeFelipe, 1993). Large cells with high proportion of cytoplasm compared

with the nucleus are found in both groups. A further criterion, which can be related to the projectional/interneuronal classification, is the density of the dendritic spines. Only the pyramidal neurons have a great amount of spines (they are also called *spiny neurons*), whereas the interneurons possess a low density or no spines at all, and are also named *smooth non-pyramidal neurons*. Finally, pyramidal and nonpyramidal cells can be distinguished by the different neurotransmitter utilised: the L-glutamate and GABA, respectively. There are also two main morphological types of synapses in the cerebral cortex; *asymmetric* and *symmetric* (Colonnier, 1968).

To date, this simple morpho-functional dichotomy has, in the last two decades, become more complicated by the fact that a number of cortical neurons contain more than one neuroactive substance, usually one "primary", conventional or classic neurotransmitter, such as GABA, and a second (or even more) chemical messenger (reviewed in Jones and Hendry, 1986). This second chemical messenger, which is commonly a neuroactive peptide, does not necessarily mimic the excitatory or inhibitory function of the primary neurotransmitter and may produce a variety of effects on its targets.

As reviewed above, recent evidence suggests that cortical projection neurons and interneurons are derived from distinct proliferative zones: the projection neurons originate from the cortical VZ and migrate toward the pial surface along the processes of radial glia; on the contrary, the majority of cortical interneurons are born in the basal telecephalon, and in particular in the basal ganglia anlage. These neurons are able to reach the dorsal telecephalon and the CP, following a tangential pathway of migration.

Most of the morphological studies on cortical neurons have been in the past carried out utilizing the rat as model. Therefore, in the following sessions, I will describe the two major cortical cell types referring to what is known for the rat cerebral cortex, considering also that there are only minor differences in overall cortical cytoarchitecture between mouse and rat.

1.10.1 Pyramidal neurons

Pyramidal cells are the projection neurons of the cortex. They constitute the largest population of cortical cells (approximately 70-85% of the total population) and are found in all layer of the mammalian cortex, with the exception of layer I.

These cells can be identified by their round to oval or triangular cell body, high cytoplasm/nuclear ratio, a round nucleus, a strong apical dendrite and several basal dendrites with numerous spines, a long axon sent to distant target, and only symmetrical synapses on their cell bodies.

Pyramidal cells are the excitatory projection neurons of the cortex and they are similar, even though they populate different cortical region, and use L-glutamate as neurotransmitter. The axons of pyramidal cells form asymmetric synapses.

There are other two types of spiny rich cortical cells that have been classified independently from pyramidal neurons: *spiny stellate cells* and *multiangular cells* of layer I (table 1.1).

The spiny stellate cells differ from pyramidal neurons having a low cytoplasmic/nuclear ratio, no clearly identifiable apical dendrite and a lower degree of dendritic polarization. Spiny rich multiangular cell, which have high cytoplasmic/nuclear ratio, have been described in layer I.

As reported above, it has been recently shown that the homebox-containing gene Emx1 is specifically expressed by pyramidal neurons in the postnatal and adult cerebral cortex of the rat, meaning that it can be used as a molecular marker of this subpopulation of cortical cells.

Spine-rich Neurons	Spine-poor Neurons
Pyramidal cells	Cells with a high cytoplasmic nuclear ratio Basket cells
Spiny stellate cells	Neuroglioform cells
Multiangular cells of layerI	Cells with a low cytoplasmic nuclear ratio Bipolar cells
	Double bouquet cells
	Chandelier cells
	Martinotti cells

Table 1.1
Neuronal cell types in the rodent neocortex.

1.10.2 Interneurons

Smooth non-pyramidal cells are short axon cells (interneurons) with smooth or sparsely spiny dendrites. They represent approximately 15-30% of the total population of neurons (Parnavelas et al., 1977; Hendry et al., 1987; Meinecke and Peters, 1987) and make up a morphological heterogeneous group of neurons (e.g., Fairén et al., 1984). Moreover these cells form symmetrical synapses and are inhibitory, utilising γ-aminobutyric acid (GABA) as neurotransmitter.

According to the different morphology of smooth non-pyramidal cells in the rat cerebral cortex, they have been subdivided into different types (Zissel, K.in the cerebral cortex of the rat, Kolb and Tees).

The *bipolar cells* have a very small cytoplasmic rim surrounding a vertically folded nucleus and two vertically oriented dendritic shafts. The cell body is fusiform, and so these cells are also named *fusiform neurons.* The axon originates frequently from one of the dendrites. Symmetrical and asymmetrical synapses are found on the soma. The dendrites are free of spines. The most typical features of the fusiform bipolar cells is the strictly vertical arrangements of dendrites and axons with a small cylindrical space.

The *double bouquet cells* are characterized by low cytoplasmic/nuclear ratio and sparsely spinous or spine-free dendrites. Like the bipolar cells, the dendrites originate from the upper and the lower poles of the cell bodies and are vertically arranged. The axon has several main vertical collaterals and arborizations in layer II through V. the cell bodies are mostly found in layers II and III.

The *chandelier cells* have low cytoplasmic/nuclear ratio, and two or more dendrites that originate from the upper and lower poles of the soma and produce tufts of ascending and descending, sparsely spinous dendrites. The axon form a laterally spread plexus and bear the boutons on the terminals in vertical strings. This arrangement gave the name *chandelier.* These boutons form symmetric synapses, whereas the cell bodies of chandelier cells have symmetric and asymmetric synapses. Most of these cells, in the rat cerebral cortex, are located in layers II *and III.*

The *Martinotti cells* of the rat are most frequently found in layers III and IV, but not excluded from more superficial and deeper layers. The soma is elongated with a low cytoplasmic/nuclear ratio. The sparsely spinous dendrites originate at the poles of the

cell bodies. The axon leaves at the upper pole or from an ascending dendrite and takes an ascending course ending with numerous terminals fanlike branches in layer I.

Finally, there are two subpopulations of nonpyramidal cells that have a high cytoplasmic/nuclear ratio: the *basket cells* and the *neuroglioform cells*. The basket cells have a round to oval cell body that can be found throughout layers II through IV; these neurons are multipolar and have smooth dendrites. The axon has branches that are oriented both orizontally and obliquely and terminates on the cell bodies of pyramidal cells. The neuroglioform cells have a small, irregularly shaped cell body with dense dendritic and axonal plexus in the immediate neighbourhood of the perikarion. These neurons have been found in layers I through VI of the rat cortex.

1.10.3 Neurotransmitters: an overview

Information carried by a neuron is encoded in electrical signals that travel along its axons and into the nerve terminals. At synapse, these signals are carried across the synaptic cleft by one or more chemical messengers.

The nervous system utilises two main classes of chemical substances for signalling: small-molecule transmitters and neuroactive peptides, which are short polymers of amino acids. Both classes of neurotransmitters are contained in vesicles; in particular, large dense-core vesicles contain the neuro-peptides, whereas small-molecule transmitters are packaged in small lucent vesicles. Most neurons contain both types of vesicles, but in different proportions.

Nine low-molecular-weight substances are generally accepted as neurotransmitters. Eight are amines, the ninth is ATP or its metabolites (table 1.2).

While the acetylcholine and the biogenic amines are not intermediates in general biochemical pathways and are produced only in certain neurons, amino acids that function as neurotransmitters are also universal cellular constituents.

The glutamate is the neurotransmitter most frequently used throughout the nervous system. After it is released, glutamate is taken up from the synaptic cleft by both neurons and glia.

Glycine is the major transmitter in inhibitory interneurons of the spinal cord.

Finally, GABA is synthesized from glutamate in a reaction catalyzed by glutamic acid decarboxylase. GABA is present at high concentration throughout the

CNS and is also detectable in other tissues, especially islet cells of the pancreas and the adrenal gland.

In general, small-molecule neurotransmitters can be formed in all part of the neuron: they can be, in particular, synthesized at nerve terminals where they are released.

By contrast, the neuroactive peptides derived from secretory proteins and they are synthesized in the cell body. In the mammalian brain, more than 50 short peptides are pharmacologically active in nerve cells. These peptides, that form a very large class of neurotransmitters with enormous diversity among them, cause inhibition or excitation, or both, when applied to appropriate target neurons.

It is to note that neuroactive peptides and small-molecule transmitters can coexist in the same neuron.

Therefore, the release of different neuroactive substances permits an extraordinary diversity of information.

TRANSMITTER	ENZYMES
Actelycholine	Choline acetyltransferase
Biogenic amines	
Dopamine	Tyrosine hydroxylase
Norepinephrine	Tyrosine hydroxylase and dopamine β-hydrolase
Epinephrine	Tyrosine hydroxylase and dopamine β-hydrolase
Serotonin	Tryptophan hydroxylase
Histamine	Histidine decarboxylase
Amino Acids	
γ-Aminobutyric acid	Glutamic acid decarboxylase
Glycine	Enzyme operating in general metabolism
Glutamate	Enzyme operating in general metabolism

Table 1.2

Small-molecule transmitter substances and their key biosynthetic enzymes.

1.11 EPILEPSY

The term *epilepsy* is derived from a Greek word meaning a condition of being overcome or seized. In modern terms, this describes the innate ability of certain types of neurons to produce a type of activity known as the paroxysmal depolarization shift (PDS), which when synchronous among many neurons causes recurrent EEG discharges as focal spikes or diffuse bilateral 1.5 to 6 Hz spike-and-wave complexes. Consciousness and senses can be abruptly suspended, and motor, sensory, or behavioral symptoms occur. The epilepsies are characterized by recurrent convulsive and nonconvulsive seizures caused by partial or generalized epileptogenic discharges in the brain.

In the past half-century, discoveries in developmental biology, neuroscience and molecular genetics have had an impact on our understanding the epilepsies.

CLASSIFICATION OF THE EPILEPSIES

Human epilepsy is a heterogeneous disorder, but to simplify, epilepsies are grouped into two categories: *partial*, in which seizure originates in a small group of neurons, that therefore constitute a seizure focus, and *generalized*, that begins without a preceding focal seizure and involves both hemispheres from the onset (table 1.3).

Classification of epileptic syndromes combines information on seizure type, age of onset, etiology, clinical course, electroencephalographic (EEG) findings.

Recurrent unprovoked seizures constitute the minimal criteria for the diagnosis of *epilepsy*. The factors influencing the seizure type and the severity can be often recognised in patterns of symptoms resulting in the identification of an epileptic syndrome. Thus a classification of the epilepsies continues to evolve, principally based on clinical observation rather than a precise cellular, molecular or genetic understanding of the underlying pathophysiology.

The primary variables are the presence of a focal brain abnormality (localisation-related) and whether there is an identifiable cause (symptomatic) or not (idiopathic).

The great majority of adult-onset epilepsies are classified as symptomatic, localisation-related epilepsies. This category includes such causes as trauma, stroke, tumors and infections.

Seizures

I. Partial (focal) seizure

A. Simple partial seizures (with motor, sensory, autonomic, or psychological symptoms)
B. Complex partial seizures
C. Complex partial seizures evolving to secondarily generalized seizures

II. Generalized seizures

A. Absence
   1. Typical (petit mal)
   2. Atypical
B. Myoclonic
C. Clonic
D. Tonic
E. Tonic-clonic (grand mal)
F. Atonic

III. Unclassified

Epilepsies (abbreviated classification)

1. Localisation-related epilepsies and syndromes
   1.1 Idiopathic with age-related onset (eg, benign childhood epilepsy with centrotemporal spikes)
   1.2 Symptomatic (eg, post-traumatic epilepsy)

2. Generalised epilepsies and syndromes
   2.1 Idiophatic with age-related onset (eg, juvenile myoclonic epilepsy)
   2.2 Idiophatic and/or symptomatic (eg, Lennox-Gastaut syndrome)
   2.3 Symptomatic

3. Epilepsies and syndromes undetermined with respect to 1and 2
   3.1 with both partial and generalised seizures (eg, neonatal seizures)
   3.2 without unequivocal generalised or partial features

4. **Special syndromes** (eg, febrile convulsions)

Table 1.3

International classification of seizures and epilepsies. (Commission on Classification and Terminology of the International League Against Epilepsy, 1981, 1985).

Understanding the epilepsy syndrome has important implications for prognosis and therapy. Unfortunately, many epilepsy syndromes do not fit in this scheme (table 1.3). One expects that this classification will be greatly refined as the criteria become based on the underlying etiologies rather than clinical observation.

IDIOPATHIC EPILEPSY

Idiophatic epilepsy lacks antecedent disease or injury to the central nervous system and is of presumed genetic origin. Moreover, the role of genetics in epilepsy is supported by the existence of several familial epileptic syndromes in humans as well as seizure-prone animal models.

The current classifications are not well correlated with genetic causes, since the same mutations can produce different syndromes in different individuals, and a single syndrome can be generated by mutations in more than one gene.

Recent studies in mice have provided insights into the molecular genetics of epilepsy. Moreover, the development of molecular markers and genomic resources has facilitated the isolation of genes responsible for rare monogenic epilepsies in humans and mice. The identified genes encode ion channels or other components of neuronal signalling.

In most cases, genetic epilepsy syndromes have complex rather than simple (Mendelian) pattern of inheritance, suggesting the involvement of many genes rather than a single one. In fact, in most forms of epilepsy the genetic influences are complex and may involve the combined effects of multiple genes and environmental factors. In addition, all genetic defects are associated with generalised seizures rather than partial or secondarily generalised seizures.

During the last two decades, progress in molecular neurobiology have led to the identification of several monogenic human epilepsy genes (table1.4). Moreover, homologous recombination in embryonic stem cells has been used for targeted inactivation (knock-out) of several thousands of mouse genes during the past decade. Some of these mutant mice, approximately 1% of the total, show spontaneous seizures (reviewed in Meisler et al, 2001).

These genes may be considered candidate genes for human disorders mapped on the same chromosome regions. If the inactivated genes are representative of the genome, several hundred genes might be targets for epilepsy mutations.

ION CHANNELS MUTATION AND NEURONAL HYPEREXCITABILITY

All the genes identified as causing idiopathic epilepsy are molecular components of neuronal signalling and the functional effect of mutant alleles provide direct evidence for neuronal hyperexcitability as one cellular mechanism underlying seizures (reviewed in Meisler et al., 2001).

The propagation of the electrical impulse in neurons is initiated by transient opening of voltage-gated sodium channels and influx of sodium ions along a concentration gradient. The impulse is terminated by the transient opening of voltage-gated potassium channels that permit the efflux of potassium and restoration of the resting potential of the cell. Voltage-gated calcium channels in the axon terminal convert the electric signal to a chemical signal via influx of calcium ions, leading to release of synaptic vesicles containing neurotransmitters. This release activates ligand-gated receptors in the post-synaptic membrane and initiates an electrical impulse in the downstream neuron. The shared domain structure of the voltage-gated potassium, sodium, and calcium channels demonstrates their evolutionary origin from a common ancestral protein. Predictions that mutations in these channels and receptors could produce disregulated neuronal firing have been confirmed by the identification of disease-causing mutations in human and mouse.

Many of the functional defects in the mutated voltage-gated and ligand-gated channels described above are predicted to increase the intrinsic excitability of neurons. Increased excitability could lead to increased neuronal firing and to episodes of synchronised firing by large numbers of neurons that constitute a seizure. The characteristics of these mutant channels strongly support this hypothesis regarding the origin of seizures.

Year	Gene	Chromosome	Mode	Types of mutant alleles	Clinical syndrome
2001	*GABRG2* $GABA_A$ Receptor	5q31	AD	Missense	GEFS+3
2001	*SCN2A* Sodium channel Alpha subunit	2q24	AD	Missense	GEFS+
2000	*SCN1A* Sodium channel Alpha subunit	2q24	AD AD	Missense null, missense	GEFS+2 SMEI
2000	*CHRNB2* acetylcholine receptor beta subunit	1p21	AD	Missense	ADNFLE3
1998	*SNC1B* sodium channel beta 1 subunit	19q13	AD	Missense	GEFS+
1998	*KCNQ2* potassium channel	20q13	AD	Missense, null	BFNC1
1998	*KCNQ3* potassium channel	8q24	AD	Missense	BFCN2
1995	CHRNA4 acetylcholine receptor alpha	20q13	AD	Missense	ADNFLE1

Table 1.4

Identified genes responsible for human monogenic idiophatic epilepsy. AD, autosomal dominant; GEFS+, generalised epilepsy with febrile seizure plus; SMEI, severe myoclonic epilepsy of infancy; ADNFLE, autosomal dominant nocturnal frontal lobe epilepsy; BFNC, benign familial neonatal convulsions.

1.11.1 Abnormal cortical development and Epilepsy

Malformations of the cerebral cortex, occurring during development, are important cause human epilepsy. In the last decade, major progress has been made in the diagnostic recognition of such malformations, especially through the use of magnetic resonance imaging (MRI).

Pathologies that affect the cerebral cortex development are usually defined as *dysplasia*; this term, therefore, is used to indicate several different developmental abnormalities.

Development of the cerebral cortex involves three distinct but overlapping processes that consist of neuronal and glial proliferation, neuronal migration and cortical organization. Cortical malformations can originate from abnormalities of any or all of these processes. Certain malformations are known to be genetically determined, while for others the genetic origin has been hypothesized. Interestingly, some cortical malformations are notably more epileptogenic than others and/or seem to be directly associated with a particular epileptic syndrome.

Abnormalities that affect cell proliferation with rare exception are also characterized by abnormal cell differentiation. Both neuronal and glial lines are usually involved and showing abnormal cell size and structure. An example of a disorder related to abnormal proliferation of cell in the CNS is *hemimegalencephaly* (HME), a malformation in which one cerebral hemisphere is moderately to markedly enlarged and structurally abnormal with thick cortex, wide convolutions, and reduced sulci. The abnormality is strictly unilateral. Laminar organization is absent in the cortex, and the demarcation between grey and white matter is poor. It has been established a close relationship between HME and epilepsy.

A malformation of the cerebral cortex which is difficult to classify is the *Schizencephaly*; it consists of a unilateral or bilateral full thickness cleft of cerebral hemispheres with consequent communication between the ventricle and pericerebral subarachnoid spaces. The walls of the clefts may be widely separated and thus be called open-lip shizencephaly., or closely adjacent and known as closed-lip schizencephaly. The clefts may be located in any region of the hemispheres but are most often found in the perisylvian area. Bilateral clefts are usually symmetric in location but not necessarily in size. Schizencephaly is associated with septo-optic dysplasia (agenesis of

the septum pellucidum and optic nerve hypoplasia) in up to one third of patients. Therefore, at the basis of this disorder could be regional absence of proliferation of neurons and glia. However, schizencephalic clefts are covered by polymicrogyric cortex and unilateral clefts are often accompanied by contralateral polymicrogyria, which could indicate a disorder of cortical organization.

Recent reports indicate that familial occurrence (Hosley et al., 1992) and a specific genetic origin due to germline mutations in the homeobox gene EMX2, are possible, at least in some cases (Brunelli et al., 1996; Granata et al., 1997). In particular, heterozygous mutations in the EMX2 gene were reported in 13 patients with Schizencephaly (Faiella et al., 1997). Severe mutations (frameshift or splicing mutations) were associated with severe bilateral schizencephaly, whereas missense mutations were associated with milder cortical abnormality.

Epilepsy is estimated to occur in 81% of patients, in equal proportion with unilateral and bilateral clefts (Granata et al., 1996). Seizures onset before the age of 3 years and seizures intractability are more frequent when the malformation is bilateral.

Disorders of neuronal migration are characterized by abnormal neuronal positioning. Several malformations, almost all highly epileptogenic, belong to this category; the most frequent finding is the presence of agglomerates of morphologically normal neurons in an abnormal site. This condition is defined as *heterotopia*. Collection of heterotopic neurons can be unilateral or bilateral, diffuse or localized, subependymal or subcortical. Seizure activity may originate both within the heterotopic cortex and the overlying cerebral cortex (Murnari et al., 1996).

Bilateral periventricular nodular heterotopia (BPNH) consists of confluent and symmetric subependymal nodules of grey matter located along the lateral ventricle, particularly along the ventricular body. Extent of heterotopia and associated with clinical symptoms are heterogeneous. BPNH is far more frequent in females, resulting in the syndrome of X-linked BPNH with prenatal lethality in males and a 50% recurrence risk in the female offspring of women with BPNH. X-linked BPNH and BPNH occurring in sporadic women has been associated with mutations of the filamin A (FLNA) gene (Fox et al., 1998).

Classical *Lissencephaly* (smooth brain) is another severe abnormality of neuronal migration characterized by absent (agyria) or thick (pachygyria) convolutions, producing a smooth cerebral surface. All patients have early developmental delay and

eventual profound or severe mental retardation. Although there are several types of lissencephaly, the most frequent and best characterized forms are those caused by mutations of the LIS1 gene (Reiner et al., 1993) and XLIS (or DCX) gene (des Portes et al., 1998; Gleeson et al., 1998).

The best known cortical malformation originating after completion of neuronal migration is *polymicrogyria*. This term designates an excessive number of small and prominent convolutions spaced by shallow and enlarged sulci, giving the cortical surface a lumpy aspect. Although polymicrogyria often is recognized only microscopically, various polymicrogyria syndromes have been identified with MRI. Bilateral perisylvian polymicrogyria appears to be the most frequent. Most reported cases have been sporadic. A few families with possible autosomal recessive or X-linked inheritance have been described. However, polymicrogyria may be unilateral, producing a syndrome of hemiparesis, mild mental retardation, and epilepsy. Almost all seizure types and several epileptic syndromes may be observed, including sleep-related electrical status epilepsy, an age-related form evolving toward spontaneous remission.

The clinical spectrum of the epilepsy associated with malformations of the cerebral cortex is broad. In theory any epilepsy which is not idiophatic could be related to underlying structural cortical changes. The relationship between the macroscopic abnormality which can be detected by MRI, underlying microscopic neuropathology, and area of seizure origin may be very complex. In addition, most of the genetically determined cortical malformations seem to be bilateral and diffuse, making it very difficult to plan surgical treatment of the associated epilepsy when antiepileptic drugs are ineffective.

Epileptogenic malformation of the cerebral cortex may affect virtually every stage of cortical development.

Moreover, the study of the most frequent cortical malformations has helped in the cloning of several genes that are critical to the normal development of the cortex and has made genetic counseling available for many families.

1.11.2 Epilepsy and the GABAergic system

Defects in GABAergic cells development and function are implicated in several common brain disorders including epilepsy.

In epilepsy, insufficient inhibitory activity predispose to uncontrolled excitatory discharges. A subset of epilepsies are associated with cortical histological abnormalities, that often have ectopic collection of neurons, which are thought to arise from neuronal migration defects (Flint and Kriegstein , 1997). It is of interest to determine whether there are specific epilepsy syndromes that are caused by disruption of development and/or function of the cells that participate in the subcortical to cortical migrations.

Over the last three decades, many studies have pointed o the lack of the GABAergic neurons as the basis of seizure activity (Charles E. Ribak et al., 1979). Since GABAergic neurons populate every cortical layer and project axons to pyramidal cell somata, they can exert an inhibitory effect on cortical projection neurons: a decrease in the number in these inhibitory neurons could lead to seizure activity of pyramidal neurons. It has also been showed that the reduction in the number of symmetrical synapses formed by GABAergic neurons at epileptic foci is mainly due to a degeneration of the terminals (Charles E. Ribak et al., 1982). Furthermore, a number of studies have indicated that there is a preferential loss of inhibitory GABAergic terminals and somata in epileptic foci (Ribak et al., 1986).

During the last years many investigators have reported changes in the GABAergic system in various models of epilepsy.

In the recent years, many studies on animal models, some of which using pharmacological approaches, have confirmed and emphasized the involvement of the GABAergic system (Lopes-Cendes I. et al., 2000) and led to the identification of mutations in genes playing roles in the signalling pathway of GABA (Stephanie Baulac et al., 2001; Robyn H. Wallace et al., 2001).

Thus, GABA is the target of many clinical antiepileptic medications. Drugs or treatments that decrease GABA-mediated inhibition are usually convulsant; conversely, drugs or treatments that increase GABA-mediated inhibition have anticonvulsant activity. Five aspects of GABA-mediated inhibitory synaptic transmission are both potential sites of epileptogenesis and also potential sites of therapeutics; these are: a) GABA synthesis; b) GABA release; c) GABA transport into cells; d) GABA type A ($GABA_A$) and e) GABA type B ($GABA_B$) receptor.

GABAergic inhibition is mediated by two major classes of receptors: type A (reviewed in Macdonald R.L. and Olsen R.W., 1994) and type B (reviewed in Bowery N., 1993).

$GABA_B$ receptors are members of the class of receptors coupled to G-proteins. They mediate a variety of inhibitory effects via second messenger cascades, and are often situated on nerve terminals where they function to inhibit neurotransmitter release.

$GABA_A$ receptors are ligand-gated chloride channels that mediate rapid inhibition. Those are the molecular targets of anticonvulsants (eg, barbiturates and benzodiazepines) as well as convulsant.

Because the ubiquity of GABA-mediated inhibition and the relevance to convulsant and anticonvulsant drug action, impaired GABA function, especially $GABA_A$ receptor function, has been related to seizure disorders. Moreover, mutations have been identified among genes for $GABA_A$ receptors; and altered $GABA_A$ receptors are observed in a variety of animal model of epilepsy.

Epilepsy is a social problem; over 50 million of people suffer from epilepsy, but only 15 million may benefit from adequate treatment. For others a proper treatment does not exist.

It is now clear that some forms of epilepsy are a "genomic" disease, and the finding of epilepsy susceptibility genes is one of the challenges of modern neurogenetics.

Chapter 2

MATERIALS AND METHODS

2.1 CHEMICALS and REAGENTS

All chemicals used are from Fluka, BDH and Sigma. Buffers were prepared according to Sambrook et al, 1989.

Restriction enzymes are from Promega, whereas Kleenow and PNK (polynucleotide kinase) from Biolabs, and T4 ligase from Roche. Rnase Inhibitor, T7, T3, and SP6 RNA polymerase are from Promega, DIG-RNA Labelling Mix from Roche, and, where it is not specified, reactions were performed according to guideline of the manufacturers.

Two types of Taq polymerase were used: Taq Promega for all genotyping PCR reactions; AmpliTaq Gold for high fidelity amplification of EMX1 exons.

Bacterial media, LB, LB-agar, SOC and SB were prepared using Difco powders according to Sambrook et al., 1989.

The reagents for immunocytochemistry and histology are from Vector laboratories and BioOptica. Substrates for chromogenic immunocytochemical reactions were from Sigma and Roche.

The glass slides are superfrost plus from BioOptica.

2.2 GENERATION OF Emx1 MUTANT MICE and ANIMAL CARE

Emx1 heterozygous mice were kindly provided by S. Aizawa (Yoshida et al., 1997).

In constructing the targeting vector, the *neo* cassette was inserted into the *Apa*I site (A) of the Emx1 gene (figure 2.1). The vector was linearized with *Not*I and introduced in TT2 ES cells derived an F1 embryo between C57BL/6 and CBA mice, by electroporation. After the screening for homologous recombinant ES clones, a mutant mouse strain has been generated.

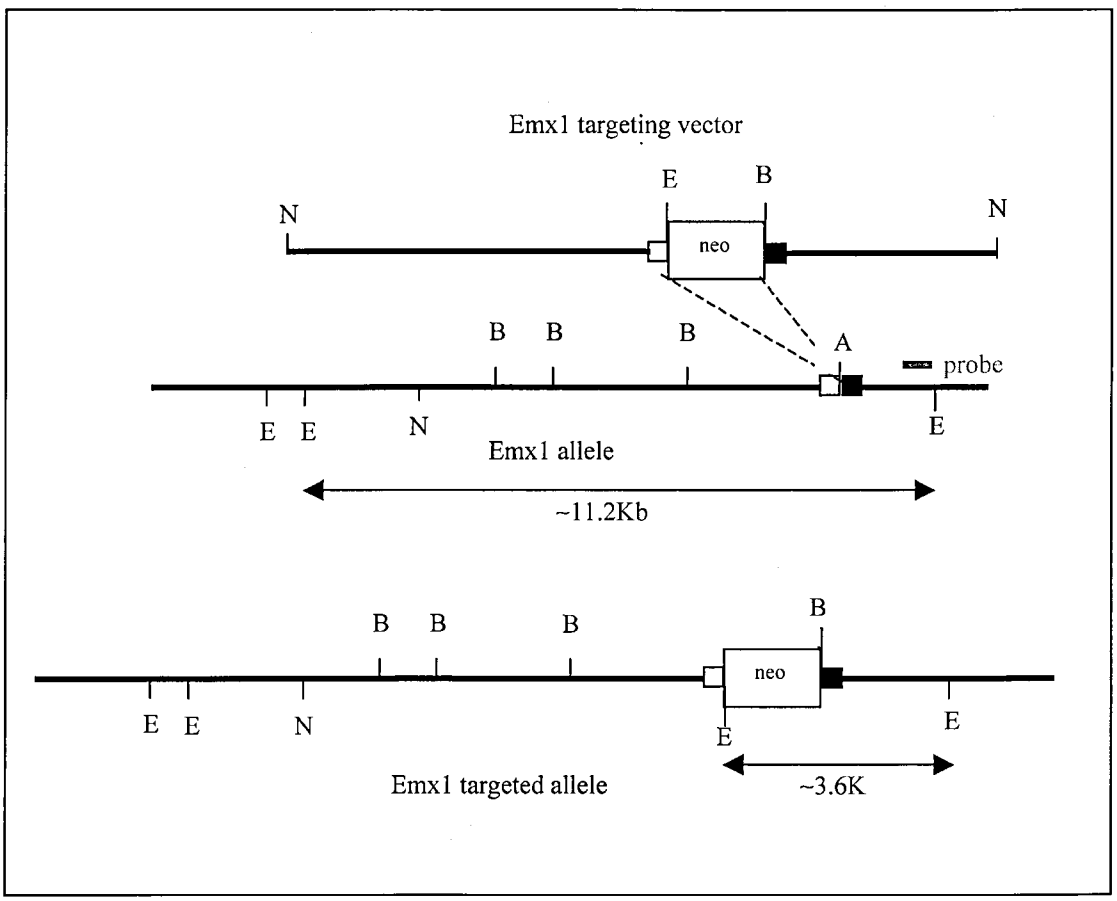


Figure 2.1
Targeting vector for Emx1 mutation. Exons are indicated by rectangles: filled box indicates the homeodomain. A, ApaI; B, BamHI; E, EcoRI; N, NotI.

One mouse male and one female, both Emx1 homozygous deficient, were generously donated by Aizawa (Yoshida et al, 1997) to our laboratory. These animals were produced by an F1 cross of a C57BL/6 female and a CBA male.

After their arrival, the animals were housed in environmentally controlled animal care rooms (SPF, specific pathogen free) of the Department of Biological and Technological Research (DIBIT), Italy, under the guidelines of I.A.C.UC. (Institutional Animal Care an Use Committee) of H San Raffaele.

In order to obtain an Emx1 mutant colony with a homogeneous genetic background, systematic backcrosses between Emx1 heterozygous animals and C57BL/6 wild type mice (Charles River, Italy) were performed.

Once the Emx1 gene inactivation was on C57BL/6 mouse strain, Emx1 mutants were obtained by intercrossing heterozygous mice. In order to collect embryos at each embryonic stage, males and females were crossed overnight and noon of the day when the vaginal plug was detected, was considered as embryonic day 0.5 (E0.5).

2.3 STANDARD DNA METHODS

2.3.1 DNA extraction from mouse tail and yolk sac

In order to obtain the genomic DNA of mice, 1 cm of the tail was cut and subsequently incubated in 500 µl a lysing buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl and 1% SDS) after adding 25 µl of proteinase K (20 mg/ml). The lysis was performed at 55°C o/n.

The DNA from mouse embryos was obtained upon extraction from yolk sac using the same protocol but a slight different lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl and 1% SDS).

The day after lysis, 35 µl of RNase A (20 mg/ml) were added and the samples are stored at 37°C, for 2 hours. At the end of this RNA degradation step, a volume of phenol is added and the tubes are kept in gently shaking for 15-20'. Then, the samples are centrifugated at 12000 rpm for 10' and the supernatant is recovered and transferred in a clean tube.

The phenol extraction is repeated again, but using a volume of phenol-chloroform, and after that a volume of chloroform-isoamilic alcohol (24:1) is added to the supernatant.

Finally, the DNA is precipitated from the supernatant with a volume of isopropanol, washed with 70% EtOH, air dried and resuspended in H_2O.

The DNA concentration can be tested on a 0.8% agarose gel.

2.3.2 DNA extraction from whole blood

Human genomic DNA of patients affected by epilepsy as well as normal individuals, was extracted from peripheral blood sample utilizing the Wizard Genomic Purification Kit (Promega), according to manufacture's instructions.

2.3.3 Recovery of DNA from agarose gel

The agarose slice containing the DNA is first cut from the gel. Then, three different strategies to recovery DNA fragments were adopted, according to the necessity.

For large amount of DNA ≥ 700-800 bp long and for the preparation of DNA probes, elettroelution is preferred: the slice is put in a dialysis tube (Gibco BRL) with running buffer and electrophoresed until the DNA is out of the gel slice. The buffer containing the DNA is then recovered from the tube and subsequently purified by phenol extraction and ethanol precipitation.

The second method is the purification through glass wool. A tiny hole is made at the bottom of a centrifuge tube with a 18G needle and a layer of glass wool is placed to cover the hole. This tube is then placed on the top of another tube, and the agarose slice, cut in tiny pieces, is layered on the glass wool. 200 µl of H_2O are then added

And the tubes are spinned for 20' @ 7000 rpm. The DNA will pass through the glass wool into the lower tube and can be purified as above.

Finally, for large-scale and quick purification of fragments to be sequenced, the QIAEX II Agarose Gel Extraction Kit (QIAGEN), was utilized, according to the manufacture's protocol.

2.3.4 Ligation in plasmid vectors

Sticky ends ligation was performed in the supplied 1X ligation buffer from Roche with 1U of T4 ligase @ 15°, for 3-4 hours.

Blunt ligation was performed in a PEG, to increase the local concentration of blunt ends (ligation buffer 5X is: 40% PEG 6000, 330 mM Tris-HCl pH 7.4, 25 mM DTT, 2.5 mM ATP), and in presence of 2-3 U of T4 ligase @ 15°C, o/n.

2.3.5 Commonly used plasmid vectors

pGEM3 (Promega)

This plasmid vector was used for cloning RT-PCR products and synthesis of RNA probes by polymerase T7 and SP6.

pBluescript SK+ (Stratagene)

This plamid vector was utilized in order to sub-clone RT-PCR products and synthetized RNA probes by polimerases T3 and T7.

2.3.6 Bacterial Strain

DH5α

Genotype: *supE44* D*lac*U169 (*f*80*lac*ZDM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1

It is a recombination-deficient suppressing strain used for plating and growth of plasmid and cosmid. The *f*80*lac*ZDM15 permits a complementation with the amino terminus of β-galatosidase encoded in pUC vectors (Bethesda Research Laboratories, 1986).

HB101

Genotype: *supE44 hsdS*20 (r_B- m_B-) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1.

It is a suppressing strain commonly used for large-scale production of plasmids. It is an *E. coli* K12 x *E. coli* B hybrid that is highly transformable (Bolivar and Backman, 1979).

2.3.7 Preparation of competent cells

DH5α and HB101 competent cells were prepared as described in Tang et al (1994).

A single bacterial colony was inoculated in 3 ml of LB broth and harvested o/n. This culture was then diluted 1:100 in 300ml of LB and shaken @ 37°C for 2h. The optical density of the culture at 600nm was monitored every 20' to follow the bacterial growth. When the O.D. reached 0.82-0.98, the growth was blocked by storage in ice 5' and from now onward everything was done at 4°C. Bacterial cells were collected by centrifugation 5' @ 3000 rpm, resuspended in 40 ml of ice cold TbpI solution (30mM potassium acetate, 100mM KCl, 10mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, pH 5.8, sterilized by filtration with 0.2 μm filter) and stored in ice 5'. Then, the cells were spinned 5' @ 3000 rpm, and the pellet was re-suspended in 4 ml of TbpII solution (10 mM Pipes, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol, pH 6.5, sterilized by filtration with 0.2 μm filter) and stored in ice for 15'.

Cell could now be aliquoted in 200μl aliquots, frozen in N_2, and stored in N_2 or -80°C.

To test the efficiency of the competent cells, 1pg, 10 pg and 100 pg of a supercoiled plasmid could be used to transform 100 μl of competent cells. A good efficiency should lead to 1-5 x 10^7 colonies/μg of DNA (10-50 colonies/pg).

LB medium (1L):

- 10g Bacto Triptone
- 5g Yeast Extract
- 10g NaCl

The pH was adjusted with NaOH, and the medium was sterilized by autoclaving.

2.3.8 Transformation of bacterial cells with plasmid DNA

Plasmid DNA in TE 1X or H_2O (1-10 ng if supercoiled DNA, 100-200 ng if a ligation reaction was used) was added to the bacterial competent cells (100 μl of an aliquot previously thaw in ice for 10').

The suspension was first mixed, and then stored in ice for 25'. Then, after the heat shock procedure performed at 37°C for 2', the suspension was incubated at room temperature for 10' . After this incubation step, 400 µl of LB were added, and the bacteria were incubated at 37°C for 45-60'.

Finally, the bacteria were plated on LB agar, additioned with antibiotics (if ampicillin is used, 50 µg/ml is enough) and the plates were stored at 37°C o/n.

2.3.9 Mini preparation of plasmidic DNA from bacteria (alkaline lysis methods)

This protocol is a slight modification of the Horowicz protocol (see Sambrook et al., 1989).

3 ml of LB supplied with the appropriate antibiotic were inoculated with a single bacterial colony and incubated o/n @ 37°C with vigorous shaking.

1.5 ml of the culture was poured into a tube and spinned 1'. The medium was aspired and the bacterial pellet was re-suspended in 100 µl of ice cold Sol I or GTE (50 mM glucose, 10 mM EDTA, 20 mM Tris.HCl, pH 8.0) and incubated 5' @ RT.

200 µl of freshly made Sol II (0.2 N NaOH, 1% SDS) were then added; the tube was inverted two or three times and kept 5' in ice.

150 µl of Sol III ($KC_2H_3O_2$ 3M respect to K and 5M respect to CH_3COOH) were added; the tube was vigorously inverted and kept 5' in ice.

Tubes were spinned and the supernatant was extract with an equal volume of buffered phenol-chloroform-isoamylic alcohol (50:49:1) and EtOH precipitated. The pellet was incubated in 50 µl of RnasiA 80 µg/ml in TE 1X 90' @ 37°C.

30 µl of PEG solution (20% PEG 6000, 2.5 M NaCl) were then added and the samples were stored 60' in ice. Subsequently they were spinned 5' and the pellet was washed in EtOH 70% before being resuspended in 30 µl of H_2O or TE 1X. The yield was around 3-6 µg of plasmid DNA per 1.5 ml of culture.

In order to obtain plasmid DNA preparations to be used in RNA probes synthesis for in situ hybridization, the use of QIAprep Miniprep Kit (QIAGEN) has been preferred; the plasmid were prepared according to manufacture's instructions.

2.3.10 Maxi preparation of plasmidic DNA from bacteria

A single bacterial colony was inoculated in 5 ml of LB broth supplied with the appropriate antibiotic and harvested o/n. This culture was then tranferred to 400 ml LB + antibiotic and harvested o/n.

Plasmid DNA was extracted using the QIAGEN Plasmid Midi and Maxi Purification Kit that is based on a modified alkaline lysis procedure and followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin.

Otherwise, the plasmid DNA was purified by using an ultracentrifuge in CsCl, as follows: the culture was first poured in Sorvall bottles and bacterial cells were collected by centrifugation for 10' @ 4000 rpm (T = 4°C), washed with 50 ml of GTE, recentrifugated as before and resuspended in 10 ml of GTE. Then, 20 ml of freshly made Sol II were added, the bottles were swirled and kept in ice for 5'. After adding 10 ml of cold Sol III, the suspension was vigorously mixed and stored in ice for 15'. Then, it was centrifugated for 15' @ 8000 rpm (T = 4°C).

The supernatant was filtered through gauze or kimwipe into clean bottles, 0.6 volumes of isopropanol were added and the solution was mixed and stored 10' in ice. Then it was spinned for 15' @ 5000 rpm, at 4°C.

The dried pellet was resuspended in 7 ml of TE 1X (the solution was warmed at 37°C), transferred to a 15 ml falcon tube and 7.5 g of CsCl were dissolved in this solution, that became ~ 88% CsCl. 300 µl ethidium bromide 10 mg/ml were added and the solution was spinned for 10' @ 3000 rpm, at 20°C (if the temperature was lower the CsCl would precipitate and the concentration would change).

The supernatant was transferred into an ultracentrifuge tube, and the tube was topped with balance solution (CsCl 88% in TE 1X). The tubes were sealed, balanced ($\Delta \leq 0.1$ mg) and spinned in a vertical rotor for 16-18 h ours @ 55000 rpm, at 20°C. The plasmid band was recovered using a large gauge (18G) needle. The sample was poured in another utracentrifuge tube, filled with the balance solution and centrifuged again 8-12 hours @ 55000 rpm @ 20°C.

The collected plasmid was extracted three or four times with isobutanol saturated with H_2O until all the ethidium bromide was removed. The sample was then dialyzed against TE 1X at least 12 hours @ 4°C.

To determine the plasmid DNA concentration, UV spectrophotometry, measuring the absorbance (A) or optic density (OD) at a wavelength of 260 nm, as well as quantitative analysis on an agarose gel, were used.

2.3.11 Genomic Southern Blot Analysis

The southern blot technique has been utilised to discriminate between Emx1 +/+, Emx1 +/– and Emx1 –/– mice or embryos, when necessary.

Genomic DNA (10-20 μg) was completely cut with EcoRI restriction enzyme (5-6 U/μg), EtOH precipitated and run on a 0.8% (w/v) agarose gel in 1X TBE buffer, together with a molecular weight marker. The gel was then photographed or the distances between the marker bands were measured.

The gel was, then, treated with 0.25 N HCl for 15' at RT in gentle shaking, washed with sterile H_2O for 3' at RT, and treated with a denaturing solution (0.4 N NaOH) for 15' at RT.

The DNA fragments were ready to be transferred from the agarose gel to a solid support (blotting): a nylon filter has been adopted (Hybond N^+, Amersham). The gel was blotted o/n in 0.4N NaOH at RT.

The day after, before to remove the filter, the positions of the gel slots and their orientations were marked on the filter with a scalpel or a pencil. Then, the filter was first treated with a solution of 50 mM Na_2HPO_4 pH 7.5, for 2' at RT, and subsequently placed on a paper towel to dry for at least 30' at RT.

The filter was then ready to be hybridized: first, the filter was incubated in a 30-50 ml of pre-hybridization solution (0.45 M Na_2HPO_4 pH 7.5, 7% SDS, 1 mM EDTA and 100 μg/ml salmon sperm DNA, which was previously denaturated at 95°C for 15'), 2 hours at 65°C.

Then the heat denatured DNA radiolabelled probe was added at the hybridization solution (the same as above) at a concentration of 2×10^6 cpm/ml, in 6-10 ml of final volume and the filter was incubated o/n at 65°C.

After the removal of the probe, the filter was washed twice in 50 ml of 40 mM Na_2HPO_4 pH 7.5, 1% SDS, for 20' at 65°C.

The filter was finally place into a transparent plastic bag without drying and exposed to a X-Ray film (Kodak) to obtain the autoradiographic image.

2.3.12 Radiolabelling of the DNA probe

The probe used for the southern blot analysis of Emx1 mutant mice was a ~700bp fragment that is shown in fig.2.1. The fragment was inserted into pBS, excised from the plasmid by digestion with XbaI, separated by electrophoresis on agarose gel, and purified from the gel. 20-30 ng of the DNA were labelled using the random primer labelling kit from Amersham and [α-^{32}P]dCTP as radioisotope to be incorporated following manufacture's instructions.

2.3.12 DNA Sequencing

DNA fragments obtained by PCR amplification of all three exons of the human EMX1 gene were sequenced either using an automatic sequencer (Applied Biosystem) or according to Sanger utilising the T7 Sequenase version 2.0 DNA polymerase kit and [α^{35}S]-dATP as radioisotope. Reactions were performed as indicated by the manufacturer.

Sequencing reactions were applied to a denaturing (urea 7M) 6% acrylamide (acrylamide:bisacrylamide 29:1) and run in 1X TBE at 65W. The gel was blotted on Whatman 3MM, dried and exposed to an X-Ray film at RT.

2.4 POLYMERASE CHAIN RECTION (PCR)

In order to define the Emx1 genotype in mice and embryos deriving from mating between heterozygous animals, PCR amplification of the normal and mutant allele has been performed. The PCR screening has been always preferred, whereas the Southern blot has been adopted when necessary.

The search of mutation in EMX1 gene in patients affected by epilepsies, has been performed by PCR. All exons and intro-exons boundary regions of the EMX1 gene in both epileptic patients and control individuals were selectively amplified by PCR.

2.4.1 Oligonucleotides

Oligonucleotide primers for the polymerase chain reaction (PCR) amplification of the mouse Emx1 wild type and corresponding mutant genomic region were as follows.

AntiEmx1 5' CTG ACA GCT CCC TAG ACA CTC TTG G 3'

SenseEmx1 5' CGT TCC CCA GGA CGG GCT GCT TTT GC 3'

SenseNeo2 5' GCC TGC TTG CCG AAT ATC ATG GTG GAA AAT 3'

A couple of oligonucleotides were also used to specifically amplify the Neomicin cassette eventually present in the mouse genome of heterozygous or homozygous mutant mice:

Neo/S 5' CTT TTT GTC AAG ACC GAC CTG TCC 3'

Neo/AS 5' CCG CAT TGC ATC AGC CAT GAT GG 3' (fragment length 213 bp)

The reaction in this case was performed at an annealing temperature (Tm) of 64°C.

As positive control for the DNA quality as well as PCR reagents and condition, an amplification reaction of the miogenin gene was run (Tm= 64°C).

MG/S 5' CCC CCA AGT TGG TGT CAA AAG CC 3'

MG/AS 5' ATG CTC TCT GCT TTA AGG AGT CAG 3' (fragment length 166bp)

The PCR analysis of EMX1 gene has been performed using several oligonucletide primers in order to amplify all the three exons and the intron-exon boundary regions of the gene. The best results of amplification for each exon were obtained using the following couples of oligonucleotides:

<u>I EXON</u>

Ex 1.1/1: 5' T CGC ACA GCT CCC GCG GCT GCC A 3'

Ex 1.1/4: 5' CG GCG GGC ACA GCG TGG ACA CTC 3'; (425 bp)

<u>II EXON</u>

EF: 5' GTG CGT GTC AAG GAA TGG AG 3'

ER: 5' GCA CGC AGT CTC GAG GAA G 3'; (461bp)

<u>III EXON</u>

Ex 1.5: 5' G CCT CCT GAG TTT CTC ATC TGT GC 3'

Ex 1.6: 5' CA CAA ACC CAC GAG GGC AGA GTG 3'; (197bp)

2.4.2 Transgenic selection by multiplex PCR

PCR amplification of mouse genomic DNA to determine the presence of the mutant allele has been performed using the following three oligos: SenseNeo2, SenseEmx1 and AntiEmx1 (see above for sequeces). TheDNA (~100 ng) was amplified by multiplex PCR in a Thermocycler (OmniGene, Hybaid). The reaction was produced in a final volume of 50 µl, and it contains:

- 5 µl of 10X PCR buffer (Promega)
- 3 µl of 25 mM $MgCl_2$ solution (Promega)
- 0.25 µl of a 100 µM solution of each primers
- 0.5 µl of a 20 mM dNTPs solution (Promega)
- 0.2 µl of Taq DNA Polymerase 5U/µl (Promega)

PCR conditions are:

1 x 3' @ 98°C

5 x 1' @ 98°C; 1' @ 64°C; 1' @ 72°C

30 x 1' @ 94°C; 1' @ 64°C; 1' @ 72°C

1 x 10' @ 72°C (this step serves to complete all polymerisation reactions)

Mineral oil (SIGMA) was added to prevent evaporation. At the end of the PCR 5 µl of 6X loading dye (Promega) were added to each tube and 20 µl of the PCR products were used for analysis in agarose minigel (1% in TBE 1X) to determine the pattern of fragments amplified.

2.4.3 Search of mutation by PCR in human EMX1 gene

The search of human EMX1 gene mutations has been performed by PCR amplification of all exons and intron-exons regions of the gene.

The amplification reaction has been carried out in a Thermocycler OmniGene, Hybaid.

The oligonucleotides utilised in this analysis, are listed above, and the reaction mixture was at a final volume of 20 µl, composed as follows:

- 100 ng of genomic DNA
- 1 X PCR buffer (Perkin Elmer)
- 1.5 mM $MgCl_2$ (Perkin Elmer)
- 0.25 mM dNTPs (Promega)
- 10 µM final concentration of each oligonucleotide
- 1U/10µl AmpliTaq Gold (Perkin Elmer)75

Amplification reactions were carried out with an initial denaturing step of 10' at 98°C, followed by 35 cycles of 1' @ 95°C, 1' @ 58 °C for exon I and II, and 64 °C for exon III, and 1' @ 72 °C

All PCR fragments were purified from the 2% (w/v) agarose gel and the sequence of both strands was determined on an automatic sequencer (Applied Biosystem).

2.5 TISSUE PREPARATION

2.5.1 Recovery of embryos and tissue sampling

Emx1 mutant embryos were obtained by intercrossing Emx1 heterozygous animals. Embryos were genotyped, upon extraction of genomic DNA from yolk sac, by Southern analysis and/or multiplex PCR.

Pregnant females at different stages of pregnancy (from E10.5 to E18.5) were anesthetized by CO_2 and killed by cervical dislocation, in compliance with European laws. Embryos were first dissected free from maternal tissues; extra-embryonic membranes were also removed. Dissection was performed in cold PBS, and the embryos were kept on ice in PBS while dissecting the whole litter.

Either entire embryos or brains that were previously dissected from the skulls, were fixed in 4% paraformaldehyde (PFA) prepared in 1X phosphate-buffered saline (PBS), pH 7.4. The fixation was allowed to proceed overnight.

After fixation was completed, the fixative was poured off and replaced with 1X PBS, to wash the samples. A total of three changes of PBS, each followed by 5' incubation at RT, was done this way.

4% Paraformaldehyde (PFA) (1L)

- warm 400ml of distilled water to 65°
- add 40g PFA
- add 40µl of 5N NaOH
- wait until the solution clears
- cool slightly and filter (0.45µm)
- add 100ml of 10X PBS
- measure pH (pH should be 7.2 to 7.6)
- bring to 1L with distilled water

WAX EMBEDDING AND MICROTOME SECTIONING

The samples were dehydrated through a graded EtOH series (from 50% EtOH to 100% EtOH) at 4°C for a variable time (30'-1 hour) according to the size of the tissue sample.

When the samples were in 70% EtOH, they could be stored, if necessary or desired. The final 100% EtOH incubation was prolonged overnight.

Then, the tissue samples were clarified by replacing the 100% EtOH with toluene. Two changes of toluene were performed in gentle shaking at RT, each followed by 30' or 1 hour incubation, depending on the tissue size,.

Then, the samples were twice or three times immerse in a toluene:wax (1:1) mixture in a 60°C oven, and subsequently transferred in 100% liquid wax at 60°C for three times, using at every step clean wax. Each of these steps was prolonged for a variable time (30'-1hour), allowing the gradual exchange between toluene and wax that can impregnate the tissue.

Finally, the sample was definitively embedded in paraffin and cast into blocks, that were left at RT to harden completely. The blocks can be stored at 4°C before to be cut. Indeed, blocks stored this way are stable and can be used successfully for years.

The sectioning was performed using a microtome and cutting serial 10µm thick sections that were subsequently mounted on Fisher SuperFrost Plus slides, treated with TESPA. A drop of 10% EtOH was put on the slides and the sections transferred onto the drop on the slide using fine brushes.

Finally, the slides were transferred into an oven or on a heating plate at 37°C.

TESPA treated slides

Slides were processed as follows:

- 10' in 10% HCl-70% EtOH
- 10' in MilliQ water
- 10' in 95% EtOH

Slides were then dried at 37°C and immersed in 2% 3-aminopropyltriethoxy-silan (SIGMA) for 20"; subsequently slides were transferred in acetone twice (10" each), rinsed in water and dried at 37°C.

OCT EMBEDDING, FREEZING and CRYOSTAT SECTIONING

After the PBS wash in order to eliminate the excess of PFA, the samples were cryoprotected by immersion in 7.5%initially, and 15% as second step, sucrose in 1X PBS, and kept in each solution at 4°C until they sank. Then the embryos or the embryonic brains were transferred to 30% sucrose in 1X PBS and stored at 4°C overnight or the time necessary to lay down in the tube.

Finally, the samples were immerse in OCT (SIGMA) and quick-frozen using isopentan cooled in dry ice or liquid nitrogen (N_2); they were then cut on a cryostat at 10-20 μm at −20/25°C. Sections were collected on SuperFrost Plus slides (BioOptica) or on gelatin coated slides and stored at −80°C for long-term storage or at −20°C if to be used soon after cut.

When cryosections were used for in situ hybridization, they were fixed with PFA and then dehydrated.

Preparation of gelatin-coated slides.

- Slides are first washed with H_2O (MilliQ), then with 100% EtOH and air dried.
- Gelatine is prepared by heating 500ml H_2O at 50°C adding and mixing 2.5g gelatin, cooling down to 30°C and adding and mixing 0.25g of KcrSO4.
- Slides are immersed in this solution for 35" and then dried at 37°C overnight.

2.5.2 Perfusion of adult mice

Perfusion was essential in adult mice in order to achieve good morphology and preservation of brains. The protocol involved first exchanging of animal's blood with phosphate-buffered saline and subsequently exchanging saline with freshly prepared ice-cooled (4°C) paraformaldehyde (PFA). A peristaltic pump was utilised, and about 5, 10 and 20 ml of 4% PFA were perfused in P0, P20 and adult mice respectively.

Essentially, mice were anaesthetized with CO_2. After the surgery to expose the heart, a needle, connected with a small diameter (1mm or less) tube immersed in PBS and passing into the pump, was inserted into the left ventricle. The right ventricle was cut, allowing the 1X PBS to be slowly but constantly perfused into the heart.

After most of the blood had been flushed out, another needle connected with 4% PFA fixative was inserted into the same puncture of the left ventricle.

After perfusion, the brain was dissected out, transferred into a tube filled with 4% PFA for post-fixation, and stored at 4°C overnight.

Further processing was carried out, as described above, by OCT embedding and freezing.

2.6 HISTOLOGICAL STAINING

The study of the development and the anatomy of the CNS in Emx1 mutant mice, was performed by staining the sections with cresyl-violet (Nissl stain), that is specific for the nervous system, and hematoxylin-eosin.

Histological stain was also used to differentiate cellular morphology after completing in situ hybridization or immunocytochemistry. Counterstaining sectioned material was necessary to positively identify the specific structures or cell types in embryos or brains. In particular, after immunocytochemistry, the hematoxylin stain was preferred to counterstain slides previously developed wit DAB or AEC. For radioactive in situ hybridized slides, the Hoechst staining of nuclei provided a fast, easy, and effective way to simultaneously view the entire tissue and the regions of hybridization.

For the histological analysis, paraffin sections were first dewaxed by two changes of xylene, 5' each at RT, and subsequently re-hydrated through the following

regimen: 100% (twice), 95%, 80%, 70% and 50% EtOH, each 5'. Finally the slides are placed in water or 1X PBS and then are ready to be histologically stained.

Frozen sections, stored at −80°C, were placed at RT to warm up and dry, and then washed in 1X PBS before use.

When histological stains were used to counterstain sections, they were already hydrated and developed.

2.6.1 Nissl Stain

In order to obtain the Nissl solution, 5g of cresyl violet were dissolved in 600ml of distilled water; 60 ml of 1M NaAcetate plus 340ml of 6% acetic acid were added. After mixing, the stain was filtered.

The slides were then incubated into the stain solution for a variable time (4-10'), which was determined for each experiment. Subsequently they were washed in distilled water, de-hydrated and incubated for 10' in the differential solution, the acid alcohol (5ml acetic acid in 200ml 95% EtOH), finally, transferred twice in xylene (5' each) and mounted in DPX.

2.6.2 Hematoxylin-Eosin

Hematoxylin-eosin stain was used to differentiate both nuclei and cytoplasm. At The hematoxylin stain used in this study was the Harris modified hematoxylin (BioOptica); the eosin powder (Sigma) was dissolved in water to make a 2% solution, stored at RT in the dark.

The slides (positioned in slide rack) were transferred into a jar filled with the hematoxylin stain, and incubated for 20" at RT. Then the slides were quickly removed and rinsed twice in water for 2' and, finally, placed into the eosin stain for 10-20" and again washed in water for 5'.

The slides were subsequently dehydrated through ascending ethanol solutions, cleared twice (5' each) in xylene, and mounted in DPX (SIGMA).

2.6.3 Hoechst Stain

Hoechst staining was performed on slides already emulsionated and developed after radioactive in situ hybridization; this staining permitted the simultaneous visualization of exposed silver grains and nuclei, allowing to determine exactly which cells have hybridized to the probe.

The slides are transferred into a 2μg/ml Hoechst 33258 solution made with distilled water (stock solution: 10mg/ml in DMSO), and incubated for 2' at RT; then, they were washed, de-hydrated and mounted in DPX.

The slides were at this point ready to be observed and photographed under fluorescence microscopy, using a red filter. Cells appeared blue, whereas the hybridization signal was detectable in red.

2.7 IMMUNOCYTOCHEMISTRY

Immunocytochemical techniques were utilised in order to localize a number of specific antigens (that are described in detail in the *Results* chapter of this thesis) into the specimen tissue. A variety of methodologies for immunocytochemical labelling of either paraffin sections or cryosections were adopted in this study.

2.7.1 Chromogenic Immunocytochemistry

IMMUNOLABELLING OF PARAFFIN SECTION

Paraffin sections were first dewaxed in xylene, rehydrated through a descending series of EtOH, rinsed in 1XPBS (as reported above for in situ hybridization).

If immunoperoxidase labellig was chosen as strategy, then, the slides were incubated in a 3% H_2O_2 solution for 5' at RT, in order to inactivate the endogenous peroxidase and, therefore, to avoid non-specific labelling. Immediately after this step, the slides were rinsed in water and transferred in 1X PBS twice (5' each) at RT.

Otherwise, if alkaline phosphatase immunoreaction was adopted the H_2O_2 step was not included in the protocol.

Subsequently, a blocking step was required in order to mask non-specific sites of binding in the tissue that could produce background signal. The blocking mixture was composed as follows:

- 1X PBS
- 10% serum (foetal calf or bovine serum, FCS or FBS)
- 1mg/ml BSA
- 0.1% Triton-X 100

Before to start blocking, the slides were removed from buffer and the excess of liquid was poured off. Then, the slides were placed horizontally (section's side up) in a slide box; PBS wet paper towels were layered in the base of the box to make a moist chamber. 500µl of blocking mix were pipetted onto slides to cover all sections. The blocking reaction was allowed to proceed for not longer than one hour at RT in the closed box.

At the end of this step, the blocking mixture was poured off and 100µl incubation mixture containing the appropriate primary antibody (monoclonal or poluclonal) were added to each slide. The optimal primary antibody concentration, where not specified by the company, was empirically determined. The antibody incubation mixture was as follow:

- 1X PBS
- 3% serum
- 0.2mg/ml BSA
- 0.1% Triton-X 100

Slides were then coverslipped with a piece of parafilm, in order to cover with a film of mixture all sections, and horizontally positioned in the box; finally they were closed and stored overnight at 4°C.

IMMUNOLABELLING OF CRYOSECTIONS

The slides were removed from the freezer, allowed to warm up to RT, rinsed in 1XPBS. If quenching of the endogenous peroxidase was required, the slides were incubated in H_2O_2 for 5' and subsequently washed in 1XPBS before to be horizontally positioned in a moist chamber.

Then, the blocking step was performed by adding 500µl of the blocking mix to each slide and incubating for one hour at RT.

Finally, the appropriate primary antibody (monoclonal or polyclonal) was added, as for paraffin section (see above).

SECONDARY ANTIBODY INCUBATION AND AMPLIFICATION OF THE SIGNAL

The following steps were performed on both paraffin sections and cryosections, without difference.

The slides were placed into a jar and washed in 1XPBS twice (5' each) at RT, blotted on paper towels and horizontally positioned into the moist slide box for the subsequent incubation with the secondary biotinylated antibody.

The secondary antibody, conjugated to biotin, was first diluted into 1XPBS; then, 100µl of the mix were added to each slide, which was subsequently coverslipped with a piece of parafilm. This incubation step was performed for 1 hour at RT. (All biotin conjugate secondary antibodies - anti-Mouse IgG, anti- Rabbit IgG, and anti-Mouse IgM, were from vector Laboratories).

About 30' before to stop the secondary antibody incubation step, the ABC reagent (VECTASTAIN Elite ABC Kit, Vector Laboratories) was prepared, following manufacture's instruction. Briefly, this system is based on the reaction that takes place between avidin (reagent A), which has four specific binding sites and biotin (reagent B), that is conjugated with the horseradish peroxidase and specifically binds to the avidin. Once the avidin-biotinylated horseradish peroxidase complex is formed, after mixing reagent A with reagent B, it retains at least one biotin binding site that will be occupied by the biotin conjugated to the secondary antibody. For each secondary antibody there will be at least three molecules of peroxidase, obtaining a strong amplification of the subsequent developed signal.

At the end of the secondary antibody incubation step, slides were twice washed in 1XPBS at RT (5' each), blotted on paper towel and horizontally re-positioned into the slide box. 500µl of the ABC reagent were added onto each slide, followed by 2 hours incubation at RT.

In order to detect the presence of low-abundance or very small molecules, that easily can flow away during tissue processing, in particular glutamate into cortical pyramidal neurons of adult mouse brains, the tyramide Signal Amplification system (TSA™, NEN Life Science Products) was adopted. This system permits a strong

amplification of the signal in standard immunocytochemistry and in situ hybridization, resulting in a significant increase of sensitivity, without loss of resolution or increase of background. The reagents and protocol were provided by the manufacture.

DEVELOPMENT

The chromogens used to localize peroxidase in tissue sections were: diaminobenzidine tetrahydrochloride (DAB) and 3-amino-9ethil carbazole (AEC).

Slides were washed twice in 1XPBS at RT (5' each). Then they were replaced into the developing solution containing the specific substrate. If DAB was utilized, the solution was prepared using DAB Tablets (Sigma) following manufacture's instruction, and the substrate produced a brown precipitate in the sections. When the desired signal intensity was reached, the reaction was stopped by rinsing slides in PBS. Finally, the sections were dehydrated through an ascending series of EtOH, being the DAB precipitate insoluble in EtOH, cleared in xylene and mounted using DPX.

Otherwise, AEC substrate produced a red reaction product in the sections and needed to be mounted in aqueous mounting medium.

ALKALINE PHOSPHATASE IMMUNOLABELLING

The alkaline phosphatase method was utilized as an alternative to the peroxidase one or for double immunocytochemistry.

The two procedures were essentially similar, but of course with some differences in the reagents utilised.

The amplification of the signal for alkaline phosphatase immunolabelling, was performed using the VECTASTAIN ABC Kit-AP (Vector Laboratories), reagent A consisting of avidin, whereas reagent B was biotin conjugated to the alkaline phosphatase enzyme.

The development was obtained adding the specific alkaline phosphatase substrates. The developing mixture was prepared avoiding light and composed as follows:

- 1ml AP-buffer (100mM Tris-HCl pH9.5; 50mM $MgCl_2$; 100mM NaCl)
- 4.5µl NBT (75mg/ml nitroblue tetrazolium (Roche) dissolved in 70% dimethylformamide)

- 3.5µl BCIP (5-bromo-4chloro-3-indolyl-phosphate (Roche) dissolved in 100% dimethylformamide).

Slides developed with NBT-BCIP substrates were dehydrated and mounted in DPX.

2.7.2 Immunofluorescent labelling of tissue sections

Immunofluorescent labelling of tissue sections was performed on frozen sectioned brains, and preferred to the peroxidase or alkaline phosphatase methods for double immunocytochemistry.

Sections were removed from freezer, and allowed to warm up to RT. Then, they were placed into the jar and rinsed in 1XPBS.

The blocking step as well as the incubation with the primary antibody was performed as for colourimetric immunocytochemistry on frozen sections.

In the case of double immunocytochemistry, after the first primary antibody (in general the monoclonal) incubation, a second primary antibody (the polyclonal) was added to slides and allowed to react for one or hours at RT.

After the incubation with primary antibody, slides were washed in 1XPBS and incubated with the secondary antibody.

When double immunocytochemistry was performed, after the two secondary antibody were added to slides sequentially: first the anti-Mouse, and then the anti-Rabbit.

The secondary antibody was first diluted in PBS and then layered on slides that were incubated in moist chamber for 1hour at RT.

The secondary antibody was conjugated to a fluorochrome. The fluorochromes utilized in this work were *fluorescein isothiocyanate* (FITC; it is excited by blue light and emits green fluorescence) and *tetramethyl rhodamine isothiocynate* (TRITC; it is excited by the green light and emits red fluorescence).

All fluorochrome-conjugated secondary antibodies utilized in this study (FITC conjugate anti-Mouse IgG and TRICT conjugate anti-Rabbit IgG) were from Southern Biotechnology Associated, Inc.

Of course two different fluorochromes were used to double-labelling the same sections for visualising two different antigens.

Finally slides were wash in 1XPBS and mounted with the Vectashield (Vector Laboratories), in order to preserve fluorescence as long as possible.

Immunofluorescent sections were observed under a microscope and/or stored at 4°C, until use.

2.7.3 BrdU immunocytochemistry

The following protocol is for visualizing BrdU (Bromodeoxyuridine) labelled cells in brain slices from animals that have been injected with BrdU (Roche).

BrdU is a modified tri-phosphate nucleoside, in particular a uridine derivative, that is incorporated in place of thymidine into the helices during the DNA replication (S phase of the cell cycle), and is therefore utilised to visualize cells that are dividing.

BrdU experiments were first performed in order to look at the cortical cell proliferation, by giving a pulse of BrdU to the pregnant animal, and killing it after 90'.

Secondly, BrdU was use to birth-date cortical cells, by injecting the pregnant dams at all embryonic stages and allow them to give pups; those were allowed to survive until the third week after birth.

BrdU PULSE-LABELLING EXPERIMENTS

Pregnant dams from E11.5 to E17.5 were injected with BrdU and after 90' from the injection killed by cervical dislocation.

Precisely, 100μg per gram of body weight were diluted in 0.9% NaCl to a final volume of 0.5ml and intra-peritoneally injected to the pregnant animal.

All embryos were chilled on ice immediately after Caesarean section. The yolk sacs were collected during the surgery and used to extract DNA for the genotype analysis. The embryo brains were fixed in 70% EtOH and 5% acetic acid overnight at 4°C. The second day, samples were dehydrated by immersion in ascending EtOH solutions, cleared in toluene and embedded in paraffin (see above for details on paraffin embedding procedure).

After microtome sectioning, 10μm thick sections were mounted on slides and dried. Before to perform BrdU immunocytochemistry, the slides needed first to be dewaxed in xylene and re-hydrated in EtOH, then washed in 1XPBS (see above) and

treated in 2N HCl for 30' at 60°C. This step was to permeabilize the nuclear membrane and to break DNA into smaller fragments that could be more accessible to the antibody.

After this step, slides were transferred into borate buffer for 10' in order to increase the pH to the physiological value, and then washed in PBS.

Subsequently, the quenching of the endogenous peroxidases was performed immersing slides in 3% H_2O_2 for 15' at RT.

A-specific binding sites in the tissue were blocked by layering 500µl of blocking mixture (1XPBS; 10% serum; 1mg/ml BSA; 0.3% triton-X 100) on slides and incubating for 1 hour at RT.

Anti-BrdU (Becton Dickinson) monoclonal primary antibody was diluted 1:50 in blocking mix and 100µl were layered on each slide, that was subsequently coverslipped with a piece of parafilm and incubated overnight at 4°C.

The second day, slides were removed from 4°C and washed twice in 1XPBS, before to start the incubation with the secondary antibody. This was a biotinyilated anti-Mouse IgG (H+L) (Vector Laboratories), and it was 1:150 diluted in 1XPBS, and incubated for 2 hour at RT.

For chromogenic detection of BrdU positive cells in the tissue, amplification with ABC and development with AEC or DAB substrates was adopted (see above for details).

Borate Buffer was prepared by mixing the following solution to obtain a pH value of 8.5:

- 4.76g of Borax in 500ml of distilled H_2O
- 3.1g of Boric Acid in 500ml of distilled H_2O

BrdU BIRTH-DATING EXPERIMENTS

Once pregnant dams, previously injected from E14.5 to E17.5, gave pups, those were allowed to grow up until postnatal days (P) P0 and P20-21 (P0 and P20-21).

The genotype of each animal was determined upon DNA extraction from the tail and multiplex PCR or Southern Blot analysis. Those animals were anaesthetized with CO_2 and transcardially perfused with 4% PFA in 1XPBS, pH 7.4. Immediately after perfusion the brains were removed from the skull and post-fixed in 4% PFA by

immersion; then, they were cryoprotected by immersion in sucrose, embedded in OCT and frozen, (see above for details on OCT embedding and freezing protocol).

After cryostat sectioning at –20°C, slides were allowed to warm up to RT and washed in 1XPBS.

The BrdU-positive cells in the tissue were detected by an immunocytochemistry procedure similar to that previously described for protein antigens (see above). The only difference consisted of the pre-treatment of sections with 2N HCl first, and borate buffer pH 8.5 subsequently, as for paraffin embedded sections (see above for details).

2.8 DETECTION OF APOPTOTIC CELLS IN TISSUE SECTIONS BY TUNEL

Nucleotide end-labelling to detect DNA fragmentation is one of the techniques for identifying apoptotic cells. The specific method utilized in this study to directly label the ends of broken DNA strands was TUNEL (TdT-mediated dUTP-biotin nick end-labelling method, based on the terminal deoxynucleotidyl transferase, TdT). TdT and biotin-dUTP were from Roche.

In this specific work, the ends of DNA fragments were labelled with biotinylated dUTP and detecting with rhodamine-conjugate streptavidin (for fluorescent detection), and the TUNEL staining was performed on frozen sections.

Slides were first removed from freezer and allowed to warm up to RT. Then, they were washed in 1XPBS, the area around the tissue was dried, and all sections were covered with 0.5% Triton-X 100 for 15' at RT.

Then slides were washed in 1XPBS, and TDT buffer was layered on each of them, followed by a 2' incubation at RT. Immediately after that, slides were washed in PBS and 150µl of TdT + biotin-dUTP (mixture prepared as follows: 1ml TDT buffer, 12,5µl TdT, 6.5µl biotin-dUTP) were layered on each slide, followed by 60' incubation at 37°C.

At the end of this incubation step, slides were washed in 1XPBS and transferred into the TB buffer for 15' at RT, and again rinsed in 1XPBS.

Samples were transferred into 2% BSA for 20'-30' at RT, washed in PBS, and finally incubated in streptavidin-rhodamine for 20'.

After the last wash, slides were mounted with coverslips and vectashield (Vector, Laboratories).

TDT buffer (for 100ml):

- 363.3mg Trizma base (30mM)
- 2.24g Na cacodylate (140mM)
- 23.8mg cobalt chloride (1mM)

TB buffer (for 100ml):

- 1.75g NaCl (300mM)
- 0.882g Na Citrate (30mM)

(Trizma and Na cacodylate are dissolved in distilled water, pH 7.2, and cobalt is then followed by water to make up a 100ml solution)

2.9 IN SITU HYBRIDIZATION

In situ hybridization to cellular RNA was utilized to determine the cellular localization of specific messages within complex cell populations and tissues. Tissue were either embedded in paraffin and sectioned on a microtome, or frozen and sectioned in a cryostat.

The RNA contained in the tissue was hybridized to a specific radiolabelled probe, which was then detected using emulsion autoradiography (Kodak).

2.9.1 Radiolabelling of Probes

The DNA templates to be used for RNA probes synthesis were cloned in plasmid vectors containing SP6, T7 or T£ RNA polymerase promoter.

The recombinant vector was linearized by digestion with the appropriate restriction enzyme, which cuts at the end of the insert opposite the appropriate promoter (or at any convenient internal site). The linearized DNA was then phenol-extracted, ethanol-precipitated and resuspended in RNase-free water (treated with DEPC).

In Vitro transcription reactions were carried out with T7, Sp6 or T3 RNA-polymerase (Riboprbe Kit, Promega), in presence of [^{35}S]CTP (Amersham).

The reaction was performed in a final volume of 30µl, at 37°C for 2 hours and the reaction mix was prepared as follows:

- 6µl of 5X transcription buffer (Promega)
- 3µl of 100mM DTT
- 1µl 40U/µl RNasine
- 4.5µl of rNTPs mix containing rATP, rGTP, rUTP ((Promega)
- 2µl 200µM rCTP
- 1µl of linearized DNA (1µg/µl)
- ^{35}SdCTP
- 2µl RNA polymerase

After RNA synthesis, the DNA template was degraded with DNaseI (Promega), and the ^{35}S-labelled RNA was purified by adding 2µl of tRNA (Stock solution 10mg/ml), 50% of the volume 7.5M ammonium acetate, 2.5 volumes of 100% EtOH and mixing. After incubating the mixture at –20°C for not longer than 30', the sample was 14000g centrifuged for 15' at RT; then, the RNA pellet was washed with 70% EtOH and resuspended by adding 50µl of 100mM DTT.

2.9.2 In Situ Hybridization

PRE-HYBRIDIZATION TREATMENT OF PARAFFIN SECTIONS

Sections were first dewaxed in xylene (2X10'), rehydrated through ethanol series (100% EtOH; 95% ETOH; 80% EtOH; 70% EtOH; 50% EtOH), washed in saline solutin (0.9% NaCl) and 1X PBS for 5' at RT.

Then the section were post-fixed with 4% PFA for 10' at RT, and washed in 1XPBS three times (5' each).

In order to denature protein and nick DNA, but also partially reverse the PFA fixation step, slides were incubated in 0.2 N HCl for 10' at RT, and after that rinsed in 1XPBS (three wash steps, 5' each).

To improve the penetration of the probe, a proteinase K digestion step was included here; the sections were incubated in 3µg/ml proteinase K (Roche) solution for 25' at RT, or, alternatively, in 20µg/ml proteinase K solution for 7' at 37°C. The proteinase K, just prior the incubation, was diluted in the specific pre-heated buffer,

composed as follows: 2M Tri-HCl pH8 and 0.5M EDTA pH 8. After this incubation step, digestion was stopped by rinsing slides twice into a 4mg/ml glycine solution in 1XPBS for 2' at RT.

The slides, at this point, were again post-fixed for 10' at RT in 4% PFA fixative, and then washed 3X 5' in 1XPBS.

The last treatment before to start hybridization, consisted of an acetylation step that blocked polar and charged groups on the tissue, which could cause non-specific electrostatic binding of the probe. The acetylation of the sections was performed as follows: slides were first transferred into 0.1M trietanolamine pH8 in constant agitation for 5', then 0.25% of acetic anhydride was added. Slides were incubated in this solution that was constantly mixed, for 5' at RT.

Slides were rinsed in distilled water and air dried, and ready to be used for hybridization.

PRE-HYBRIDIZATION TREATMENT OF CRYOSECTIONS

The pre-hybridization treatment of cryosections differs from that of paraffin sections.

Frozen sections, stored at −80°C, were removed from freezer and allowed to come to RT. Then, the slides were rinsed in 1XPBS, post-fixed in 4% PFA for 10' at RT. After rinsing slides in 1XPBS, they were de-hydrated by EtOH ascending concentration solutions, and air-dried. The following steps were performed as for paraffin sections.

HYBRIDIZATION

Hybridization and all subsequent steps were performed following the same procedure on paraffin sections and cryosections.

The appropriate labelled riboprobe (sense or antisense) was added to the hybridization mix to obtain 6×10^6 cpm per each slide, and the mixture was vortexed.

The following hybridization mixture was utilized:

- 50% formammide
- 10% (w/v) dextran sulphate
- 500µg/ml tRNA [Ribonucleic Acid Transfer type X-SA (Sigma)]
- 50mM DTT

- polyadenylic acid (5') (Sigma)
- 1X salts solution (10X solution as follows: 3M NaCl; 100mM Tris-HCl pH 7.5; 100mM NaH_2PO_4 pH 6.8; 50 mM EDTA pH8; 2% Ficoll 400; 2% polyvinyl pyrrolidone; final pH 7.2).

The hybridization mixture was applied to the dry slides by depositing 50-100 µl in the centre of the area containing the tissue sections. Each slide was covered with a piece of parafilm pre-cut in order to cover the sections.

The slides were then placed in an incubation box containing 50% formammide, 25% of 20XSSC and 25% sterile water, to produce a moist chamber. This box was sealed and placed in a water-bath at 65°C overnight.

POST-HYBRIDIZATION WASHES

The second day, slides were first dipped in into room temperature 5X SSCto quickly remove coverslips, and then transferred into clean 5XSSC and incubated for 30' at RT.

The slides were then sequentially treated with:

- 50% formammide for 30' @65°
- NTE solution for 15' @ 37°C (twice)
- NTE solution-20µl/ml Rnase A for 30' @ 37°C (these treatment was utilised to degrade all imperfect RNA-RNA hybrids
- NTE solution for 15' @ 37°C
- 50% formammide for 30' @ 65°C
- 2X SSC for 15' @ RT
- 0.1X SSC for 15' @ RT

The slides were dehydrated by passing them successively for 1' in 50%, 70%, 80%, and 95% ethanol (each containing 0.3M ammonium acetate), followed by absolute ethanol. Once dried, the slides were exposed overnight against film for autoradiography at RT. This low-resolution autoradiography is not sufficient for analytical purpose but it is very useful to predict the time required for emulsion autoradiography, which was adapted for each individual probe.

NTE solution:

- 0.5M NaCl
- 10mM Tris-HCl, pH 8
- 5mM EDTA, pH 8

DEVELOPMENT

Development was performed in the darkroom. Emulsion autoradiography was performed using Kodak NT/B2 emulsion.

The emulsion was first diluted in the dark by adding an equal volume of distilled water, warming both for 30' in a water-bath at 40-45°C; the diluted emulsion was then stored in small aliquots at 4°C and protected from light.

Before use, an aliquot of emulsion was incubated at 45°C for 20' and then poured into a dipping chamber placed in the 45°C water-bath. The slides were then dipped by immersing them slowly for about 3" in the emulsion, and then removed and blotted for a few seconds vertically on a stack of paper towels.

The slides were allowed to air-dry, and then were placed into a light-tight box containing silica gel. This box was subsequently closed, sealed and covered with aluminium foil, and exposed at 4°C. The exposure time was always between 6 and 12 days.

When ready to be developed, the box containing the slides was first allowed to warm up to RT. Then, in the dark, the slides were placed into the developer (Kodak D19, 160mg/l) for 2', rinsed in tap water (30") and transferred into the fixer (Kodak AL 4, 0.2% v/v in water) for 3' and again rinsed twice in tap water.

At this point the light could be turned on and the slides processed for counterstaining (see above for details).

At the end, the slides are dehydrated and mounted with DPX.

2.9.3 In Situ Hybridization and Detection Using Non-isotopic Probes

Non-radioactive in situ hybridization was used to determine the cellular location of specific transcript within the tissue. The RNA in the tissue was hybridized with a probe labelled non-isotopically with digoxigenin; the probe was subsequently detected by an enzyme-linked immunoassay using an antibody conjugate. In particular, in the

case of digoxigenin-labelled probes this involves incubation with anti-digoxigenin antibody coupled with alkaline phosphatase, followed by a colour reaction catalysed by the alkaline phosphatase. The substrate for the alkaline phosphatase used in this work were the nitroblue tetrazolium salt (NBT, Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche).

For non-isotopic in situ hybridization to either paraffin sections or cryosections , the slides were prepared as already reported for radioactive in situ hybridization (see above).

The digoxigenin-labelled probes were prepared as follows:

- 1-2µg of the appropriate linearized vector
- 2µl RNA polymerase (Promega)
- 4µl of 5X transcription buffer
- 2µl of 100mM DTT
- 1µl Rnase inhibitor (Promega)
- 2µl of DIG RNA labelling mix (Roche)
- RNase-free water to a total volume of 20µl

The transcription reaction was performed overnight at RT.

The day after the RNA probes were precipitated adding to the reaction mix 18µl of 6M ammonium acetate, 2.5 volumes of EtOH and 2µl of 10mg/ml tRNA , and incubating at –20°C for 20'. After centrifugation, the pellet was washed with EtOH and resuspended in 50µl of 100mM DTT.

HYBRIDIZATIONAND POST-HYBRIDIZATION WASHES

Once slides were pre-treated, the hybridization step followed. The DIG-labelled RNA probe was diluted into the hybridization mixture (the mix was the same as for radioactive in situ hybridization, see above for details) to a final concentration of 200-400µg/ml.

100µl of the mix were added to each slide that was subsequently coverslipped with a piece of parafilm and incubated at 65°C overnight in a moist chamber.

The following day, slides were washed according to the protocol reported above for radioactive in situ hybridization.

IMMUNOCYTOCHEMICAL STAINING

After the last wash with 0.1X SSC, slides were transferred into B1 buffer and allowed to equilibrate for 5' at RT.

Subsequently, each slide was horizontally positioned into a humidified slide box, covered with 500µl of B2 solution, and incubated for 1 hour at RT.

At the end of this incubation step, B2 solution was replaced with 500µl of 1:500 anti-digoxigenin antibody (Roche) in B1 + 1% serum, and slides were incubated overnight into the moist chamber placed at 4°C.

The following day, slides were first washed three times with B1 buffer (5' each at RT), and subsequently equilibrated with B3 buffer for 5'.

Finally, 500µl of B4 solution were put on each slide, for the development of the chromogenic reaction. This reaction was stopped at the desired signal intensity by rinsing slides in 1X TE, pH8.

Slides were then dehydrated and mounted with DPX.

B1 buffer (TBS):

- 0.1M Trsi-HCl, pH7.5
- 0.15M NaCl

B2 solution: B1 +10% serum

B3 buffer:

- 0.1M Tris-HCl, pH9.5
- 0.1M NaCl
- 50mM $MgCl_2$

B4 solution:

- 3.5µl BCIP (5-bromo-4chloro-3indolyl-phosphate) (Roche)
- 4.5 NBT (nitroblue tetrazolium) (Roche)
- 1ml of B3 buffer

2.10 DiI TRACING

Embryos were fixed by immersion in 4% paraformaldehyde in PBS. Brains were removed from the skulls and injected with DiI (large crystal preparation).

Single DiI crystals (100-200 μm) were placed in the cortex of one hemisphere (usually the presumptive somatosensory region) and in the dorsal thalamus of the other hemisphere, using an insect pin.

After storage at room temperature in fixative for 4 weeks in the dark, brains were rinsed in PBS, embedded in 4% low melting point agarose in PBS, and cut coronally at 100 μm on a Vibratome. Sections were counterstained with DAPI (Molecular Probes), coverslipped in 30% sucrose in PBS, and examined by epifluorescence microscopy.

The number of DiI cases studied at each age is shown in table 2.1.

	CORTICAL INJECTIONS	THALAMIC INJECTIONS
E 14.5	4 +/+ or +/-; 4-/-	4+/+ or +/-; 4-/-
E15.5	6+/+ or +/-; 6-/-	6+/+ or +/-; 6-/-
E16.5	4 +/- or +/-; 4-/-	4+/+ or 4+/-, 4-/-
E17.5	7+/+; or +/- 7-/-	7+/+ or +/-; 7-/-
E18.5	4+/+ or +/-; 4-/-	4+/+ or 4+/-; 4-/-

Table 2.1 Number of brains studied by DiI injection at different embryonic stages between E14.5 and E18.5.

2.11 ELECTROENCEPHALOGRAFIC ANALYSIS

2.11.1 Surgical procedure for electrodes implantation

Emx1 null mice (N=8) and wild type (N=8) littermates were anaesthetized with Equithesin (1% phenobarbital/4% choral hydrate, Sigma; 3.5 ml/kg, i.p.) and nichrome insulated bipolar electrodes were implanted in the right hippocampus (coordinated from bregma: (mm) (nose bar 0); AP - 1.9; L ± 1.5; 1.5 below dura) and contralateral parietal cortex (coordinated as above except for depth, 0.3 mm below dura) and a ground lead was positioned over the nasal sinus (Vezzani et al., 2000).

The electrodes were connected to a multipin socket and secured to the skull with acrylic dental cement. Mice were allowed 3-5 days to recover from the surgical procedure before the start of the study.

2.11.2 Electroencephalographic (EEG) recordings

EEG recordings were carried out in freely-moving mice according to Vezzani et al, 2000. A baseline recording was done in wild-type and knock-out mice to assess the spontaneous EEG pattern. The EEG recordings were made twice a day (between 9 and 12 am, and 4 and 6 pm) continuously for at least 30'. Mice were recorded 3 times per week for two consecutive weeks.

EEG from each animal was visually analysed by two independent investigators unaware of the identity of the experimental groups. Particular attention was paid to the occurrence of ictal episodes (high frequency and/or multispike complexes and/or high voltage synchronised spikes simultaneously occurring in both leads of recordings) and/or spiking activity.

2.11.3 C-fos Immunocytochemistry

At the end of the last recording session, mice were transcardially perfused with 4% paraformaldehyde (see above). Immunocytochemistry was carried out using 35μm freely floating coronal brain sections cut with a cryostat at −20°C throughout the forebrain (from plate 20 to plate 54; Atlas of the mouse brain and spinal cord, Sidman et al., 1971). Slices were incubated with the primary antisera at 4°C for 48h by using a

polyclonal anti human c-fos antibody (1:10000; Oncogene Res Prod). After washing in PBS, immunoreactivity was tested by the avidin-biotin-peroxidase technique (Vectastain ABC kit; Vector Laboratories). The sections were then reacted with 0.4mM 3'-3'-diaminobenzidine (Sigma) washed in PBS, dehydrated, coverslipped in DPX, and observed at the light microscope.

2.12 DETECTION AND QUANTIFICATION OF AMINO ACID NEUROTRANSMITTERS IN ADULT BRAINS

In order to simultaneously determine the total and the released quantity of the amino acid neurotransmitters in Emx1 mutants, a sensitive high performance liquid chromatography (HPLC) method was adopted.

Various derivatization methods exist for the conversion of amino acids to detectable forms; in the present study, the fluorimetric detection of primary amino acids treated with o-phthaldialdehyde (OPA) in aqueous solution was chosen according to Fedele et al. (1998).

The reaction yields isoindole derivatives of the primary amino acids, rendering them highly fluorescent. Once injected into the HPLC system, the derivatized sample is carried at constant flow rate through the chromatography column in an optimized buffer, which constitutes the mobile phase. Bonded to the matrix of the column are long carbon chains (C_{18} column), which form a hydrophobic stationary phase. Separation occurs as the derivatized amino acids in the sample are retained in the column on the basis of their relative hydrophobicity in the mobile phase. The fluorescent detector can measure picograms quantities of the OPA-derivatized amino acids as they elute from the column.

Six adult (8 weeks old) Emx1 wild type and the same number of Emx1 null mice were sacrificed by decapitation and occipital cortices quickly dissected out at 0°C. Crude synaptosomes were prepared as described in Raiteri et al., 1984.

The tissue was homogenized in 40 volumes of 0.32M sucrose (pH 7.4 with phosphate buffer) using a glass-teflon grinder (clearance 0.25mm). The homogenate was centrifuged (5 min, 1,000 x g at 0-4°C) to remove nuclei and debris, and synaptosomes isolated from the supernatant by further centrifugation (20min, 12,000 x g at 0-4°C). The synaptosomal pellet was then resuspended in a standard medium

having the following composition (mM): NaCl, 125; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 22; glucose, 10 (aeration with 95% O2 and 5% CO2); pH 7.4. Synaptosomes were incubated 15 min at 37°C under gentle shaking. Identical aliquots of the synaptosomal suspension were distributed on microporus filters placed at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri et al., 1974) and superfused with continuously aerated standard medium (see above) at a rate of 0.5ml/min for a total time of 48h.

After a 36 min stabilization period, consecutive samples were collected according to the following scheme: basal release (B1; 3'); K^+ -evoked release (S; 6'); after depolarization basal release (B2; 3').

Synaptosomes were exposed to the depolarizing stimulus (50mM KCl replacing an equimolar concentration of NaCl) for 90", starting at the end of the first fraction collected (B1). Endogenous amino acids content in each sample was determined by HLPC analysis using fluorimetric detection. The K^+ -evoked overflow was estimated by subtracting the total basal release (B1+B2) from the total K^+ -evoked release (S).

Differences have been analysed by ANOVA followed by two-tailed Student's t-test and considered significant at the level of $p<0.05$, at least.

Chapter III

RESULTS

Little is known about the molecular mechanisms underlying the complex changes that occur during the development of the forebrain, and about the genetic regulation of this process.

Emx genes have been shown to be expressed in the developing forebrain. In particular, *Emx1* shows the most widespread expression both in proliferating and post-mitotic neurons of the cerebral cortex. Moreover, the gene expression in the cerebral cortex starts during the embryogenesis and persists in adult life. This spatial and temporal expression profile suggests a possible involvement of *Emx1* in the control of proliferation, migration and differentiation of cortical neurons as well as in the establishment and maintenance of connections, electrical circuits, and specificity of cortical areas.

Emx genes have been inactivated in mouse. Over the very last years, many interesting and useful data have become available about the several functions played by Emx2, making this gene an eclectic protagonist at all stages of cortical development.

By contrast very little is known about the role of *Emx1*. Previous reports have shown that *Emx1* null mutant mice exhibit very subtle structural alterations of the brain.

We decided, therefore, to study *Emx1* loss of function mice in order to elucidate the functions that this gene might play in the formation of the cerebral cortex and its functionality.

3.1 Emx1 LOSS OF FUNCTION MICE

Two Emx1 homozygous mutant mice were kindly provided to our laboratory by S. Aizawa (Yoshida et al., 1997). Those animals were derived from a backcross between a C57BL/6 Emx1$^{+/-}$ male with a CBA Emx1$^{+/-}$ female.

3.1.1 Transgenic mice selection

The three Emx1 genotypes (homozygous mutant, heterozygous and homozygous normal) were determined utilising both Southern Blot analysis and/or multiplex-PCR (for further details, see Materials and Methods). DNA was extracted from tail for the post-natal and the adult mice selection, and from yolk sac for the embryos. Figures 3.1*a* and *b* show examples of the results obtained by PCR and Southern respectively.

3.1.2 Backcrossing on C57BL/6 genome

We obtained Emx1 mutant mice with C57BL/6 genetic background, by backcrossing Emx1 heterozygous animals with C57BL/6 wild type mice (Charles River, Italy). The results of this procedure are summarised in figure 3.2.

We assumed that the genome was 100% C57BL/6, after the tenth backcross.

Once the Emx1 gene mutation was in the C57BL/6 genome, to define the phenotypical consequence of removing Emx1 gene in vivo, we interbred Emx1 heterozygous mutant litters, to generate homozygotes.

3.1.3 Influence of the genetic background on the formation of the corpus callosum in Emx1$^{-/-}$ mice

Previous studies reported that Emx1$^{-/-}$ mice are viable and display very slight defects restricted to the forebrain (Qiu et al., 1996;Yoshida et al, 1997). In particular, the most evident abnormality in the mutants was the absence or defasciculation of the

corpus callosum, which is the main fiber track connecting the left and right cerebral hemispheres.

According to Qiu et al (1996), in the absence of a functional Emx1 gene, 100% of Emx1 homozygous mutant mice, which contain a significant portion of background derived from the 129-strain, lack most or all of their corpus callosum On the other hand, Yoshida et al. (1997) found that their Emx1$^{-/-}$ mice, with a mixed C57BL/6 and CBA background, exhibit an abnormal corpus callosum, but just in a percentage of cases. All wild type controls were normal.

To date, none of these groups of researchers have reported other significant brain abnormalities in Emx1 mutant mice.

It has recently come to light that the genetic background could influence the outcome of the corpus callosum defects in mutant mice generated by EC cell technology.

To determine whether the corpus callosum abnormalities in Emx1 mutant mice were a consequence of the gene inactivation itself or a genetic background effect, we analysed the embryonic and the post-natal brains of Emx1$^{-/-}$ mice and wild type littermates. We found that the corpus callosum was always present (the total number of Emx1$^{-/-}$ embryos, post-natal and adult brains analysed was between 70 and 80), and just in a very few Emx1$^{-/-}$ brains it was smaller and defasciculated.

As a confirmation of our data, Guo et al. (2000) have published results from a similar analysis showing that C57BL/6 Emx1-/- mice develop a normal corpus callosum.

These data together indicate that Emx1 gene does not contribute directly to the defects of the corpus callosum.

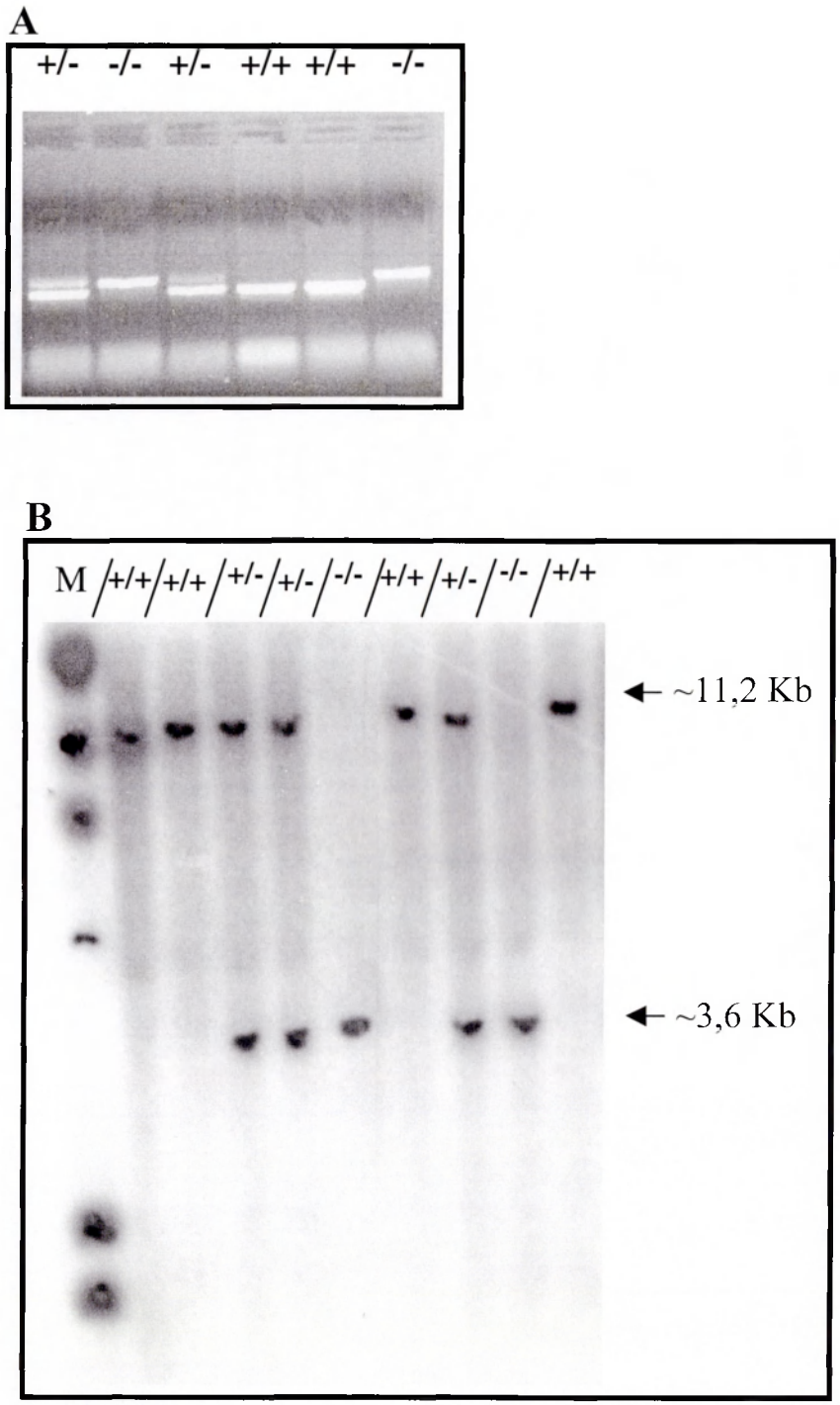
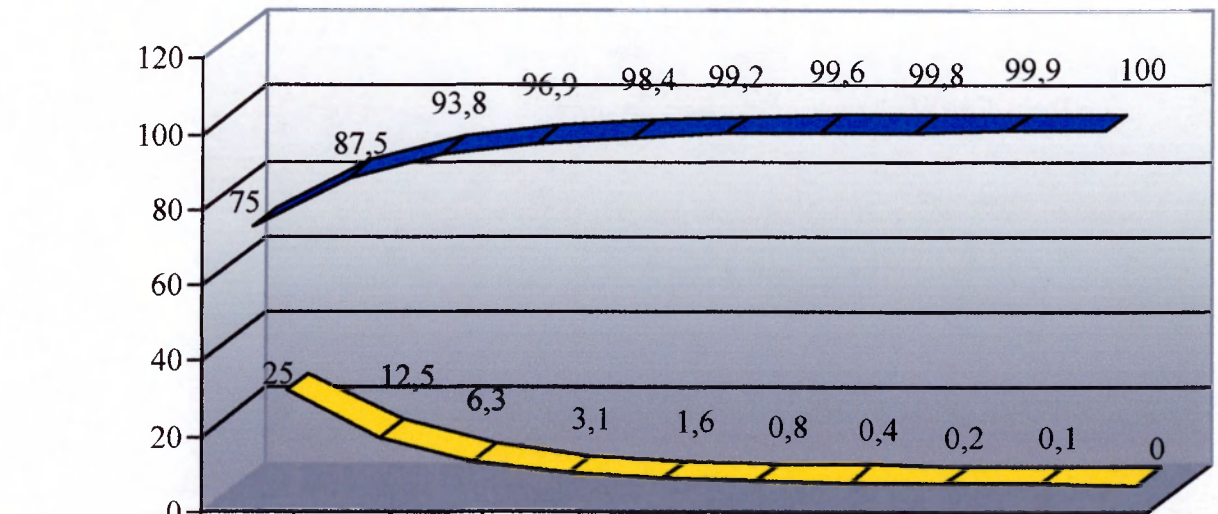


Figure 3.1

Example of multiplex PCR (A) and Southern blot analyses (B) of the Emx1 genotype in mice. (B) Southern blot was performed by *EcoRI* digestion of genomic DNA. The probe utilised and the genomic region is shown in figure 2.1. (M, molecular marker; λ DNA digested with HindIII; +/+, homozygous normal; +/- heterozygous; -/- homozygous mutant).



	1	2	3	4	5	6	7	8	9	10
% C57BL/6	75	87,5	93,8	96,9	98,4	99,2	99,6	99,8	99,9	100
% CBA	25	12,5	6,3	3,1	1,6	0,8	0,4	0,2	0,1	0

Figure 3.2 Schematic representation of the progression of the murine CBA genome toward the C57BL/6 genome, according to the number of backcross.

3.2 NORMAL NEOCORTICAL DEVELOPMENT IN EMX1$^{-/-}$ MICE

We performed systematic morphological and molecular analyses to investigate if the absence of a functional Emx1 gene would affect the development of the CNS, and in particular of the cerebral cortex in mouse. In order study the development of Emx1$^{-/-}$ mice and determine if even subtle alterations might occur, we first used standard histological methods. Subsequently, specific molecular markers were utilised to dissect the molecular changes and the anatomical consequence of the absence of a functional Emx1 gene.

3.3.1 Histological analysis of Emx1$^{-/-}$ mice throughout embryonic development

We, initially, decided to visualise the phenotypic effects of the mutation during embryonic development, utilising standard histological methods. Therefore, we harvested Emx1$^{-/-}$ and wild type embryos at all embryonic stages from E10.5 to birth (P0). In order to get a global outlook at the developing brain, sections in different planes (sagittal, coronal and frontal) were cut and subsequently stained.

We started the analysis from E10.5, when the two telencephalic vesicles first appear, and one day after the onset of Emx1 gene expression. We initially noted that the Emx1$^{-/-}$ developing brains (and in general embryos) were indistinguishable from the Emx1$^{+/-}$ and Emx1$^{+/+}$ ones.

Subsequent events of CNS development occurred also normally in the mutants: the telencephalic vesicles separate from each other and expand, whereas the neuroepithelium (figure 3.3), that forms the cerebral cortex in the dorsolateral portion of the expanded telencephalic vesicles, starts to be delineated without disturbances.

At E13.5, the mutant phenotype was still undetectable by morphological examination of the embryos (figure 3.3, B). Until this stage, the telencephalic vesicles undergo a demarcation into a "roof", the rudiment of the cerebral cortex, and a "floor", the rudiment of the basal ganglia. The germinal cells in the cortical neuroepithelium are still prominent components of the cerebral cortex, but cortical lamination starts to evolve (figure 3.3, B).

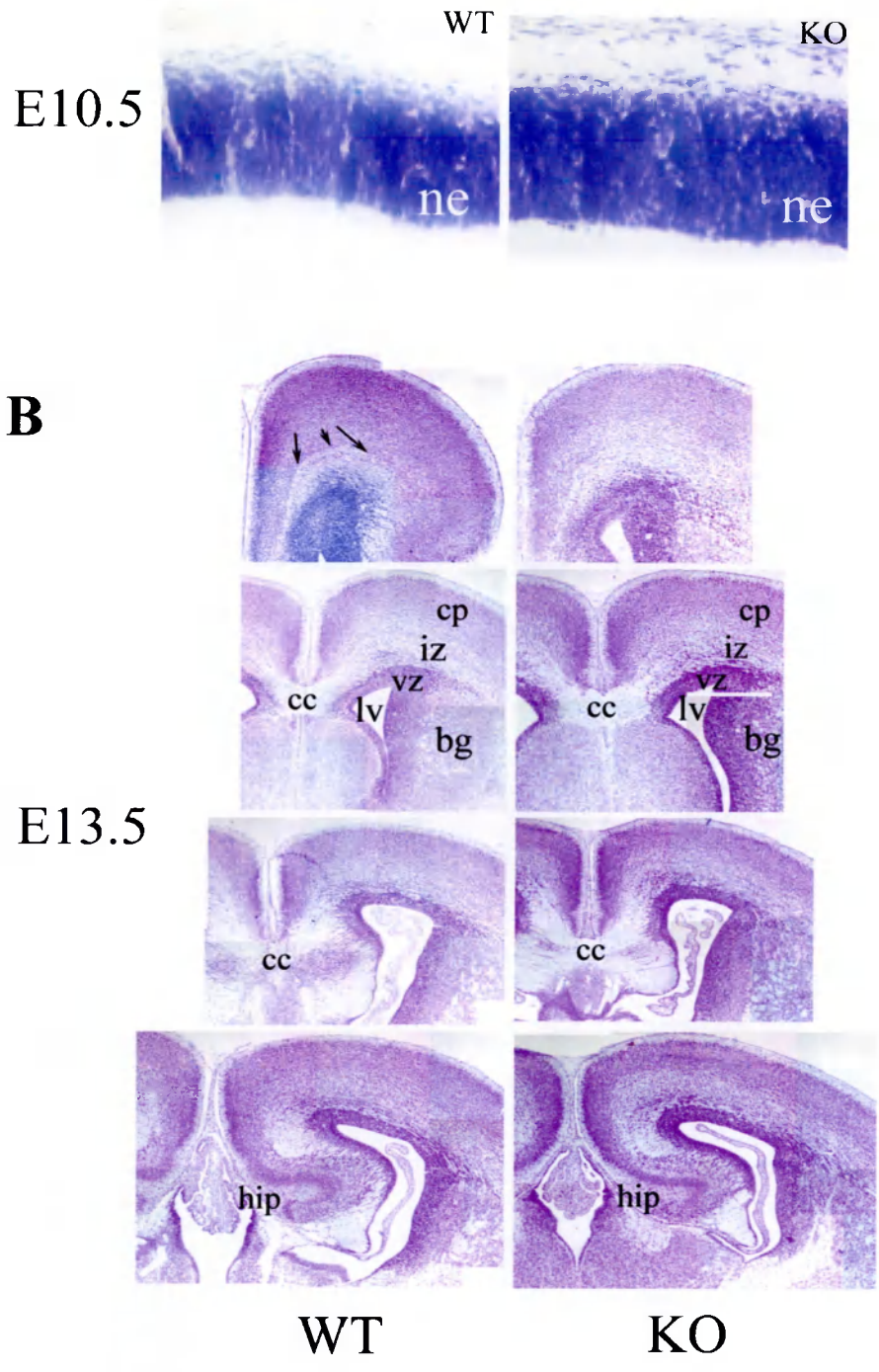


Figure 3.3

Nissl-stained sections of E10.5 (A) and E13.5 (B) Emx1 knockout and wild type embroys. A, high magnification view of the dorsal telencephalon germinative neuroepithelium of a E10.5 wild type and a mutant embryo. B, comparison between E13.5 frontal sections of a normal brain (wt) and a mutant one (ko) from rostral to caudal. (cc, corpus callosum; hip, hippocampus; bg, basal ganglia (primordia); lv, lateral ventricle; vz, ventricular zone; iz, intermediate zone; cp, cortical plate)

Later during mouse development, we still could not detect any abnormality comparing Emx1 deficient embryos with the wild type littermates. Around E15.5, in wild type dorsal telencephalon as well as in the mutant one, the cortical layering becomes more evident with the appearance of the transitional field at the expense of the germinal neuroepithelium (figure 3.4). The transitional field (TF) contains the SVZ and the IZ, which are either present only embryonically or are substantially transformed in the adult cortex, and the cortical plate (CP). Moreover, we could easily detected at this stage that in $Emx1^{-/-}$ embryos callosal axons have started to fasciculate forming the corpus callosum (figure 3.4). The archicortex also begins to be clearly patterned.

Even at later embryonic stages, the development of $Emx1^{-/-}$ embryos did not seem to be perturbed. At E17.5, for example, wild type and mutant brains were practically identical (figure 3.5): the thickness of the neuroepithelium has decreased by this day, whereas the width of differentiating cell layers has increased appreciably. The presence of a normal corpus callosum was always found in mutants, as well as a morphologically normal hippocampal formation.

We never detected any abnormalities in the developing olfactory bulbs.

According to the previous reports, this morphological study suggested that CNS development was not perturbed by the deletion of Emx1 gene. As a confirmation, we could observe a generally normal neuroanatomy in P0 $Emx1^{-/-}$ brains (figure 3.6).

However, the only difference that we could visualise between the two groups of animals was at the level of the subplate. Although not in all samples, but specifically in less than 50%, the subplate was hardly visible in the Emx1 null cortex (see pictures G and H in figure 3.6).

3.3.2 Immunocytochemical study of the developing $Emx1^{-/-}$ cerebral cortex

To follow the molecular changes occurring during neural development, the expression of molecular markers such as Emx2, TuJ1, Map2, GAP-43, CR, GFAP, vimentin and Tbr1 was analysed on sections from E12.5 to E19 embryos, both Emx1 null and wild type littermates. Our intention was to "dissect" processes of neocorticogenesis, such as proliferation, migration and settling of cortical cells

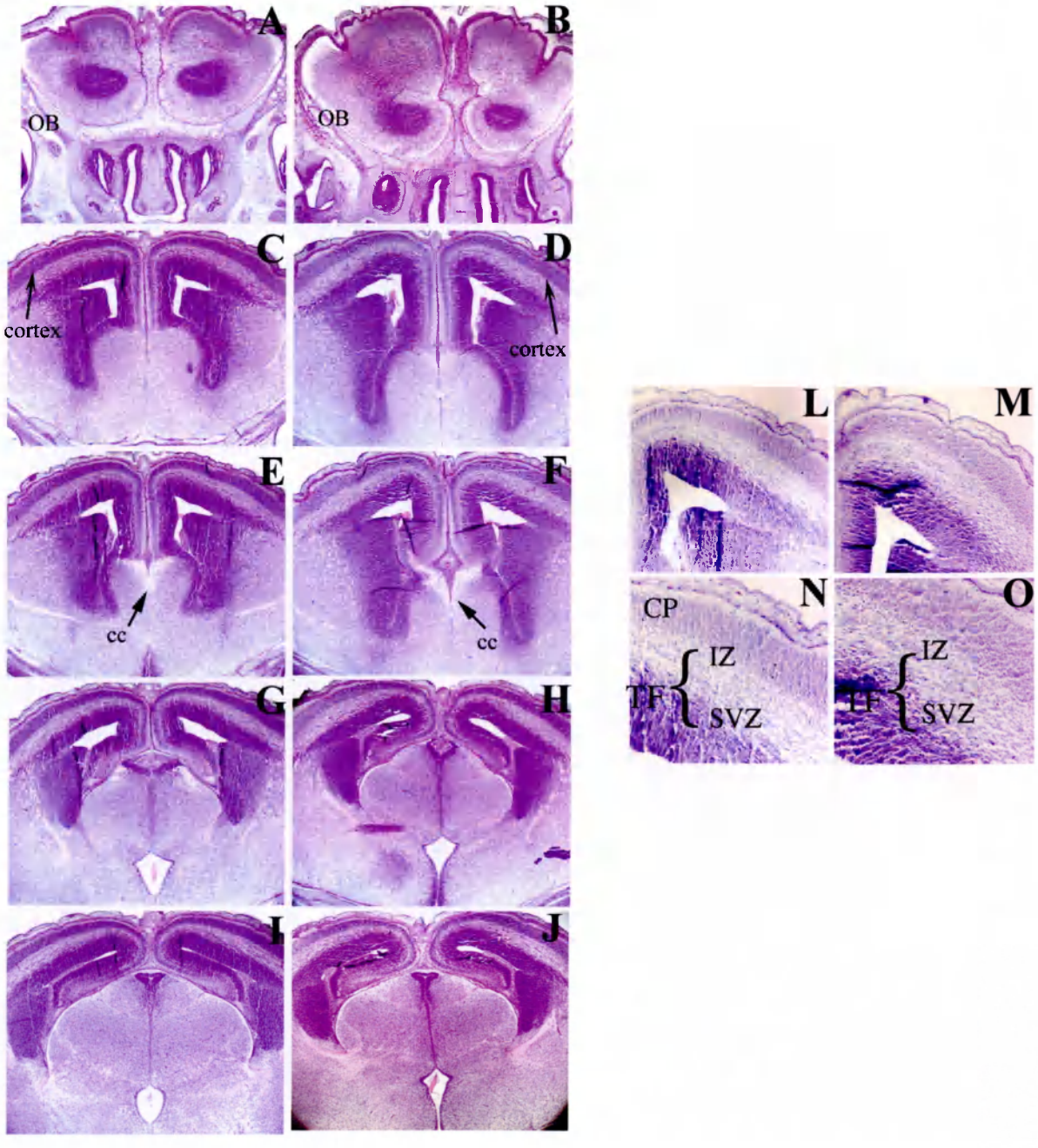


Figure 3.4

A-J, Hematoxilin-eosin stained E15.5 frontal sections of wild type (A, C, E, G, and I) and Emx1 null (B, D, F, H, J) embryos. L-O, Nissl-stained E15.5 frontal sections of a wild type (L and N) and a mutant (M-O) embryos. In N and O, the developing cortical plate (CP) as well as the transitional field (TF) can be easily distinguished both in the mutant and the wild type. The transitional field is composed by the subventricular zone (svz) that lies just above the germinative neuroepithelium, and the intermediate zone, located below the forming cortical plate.

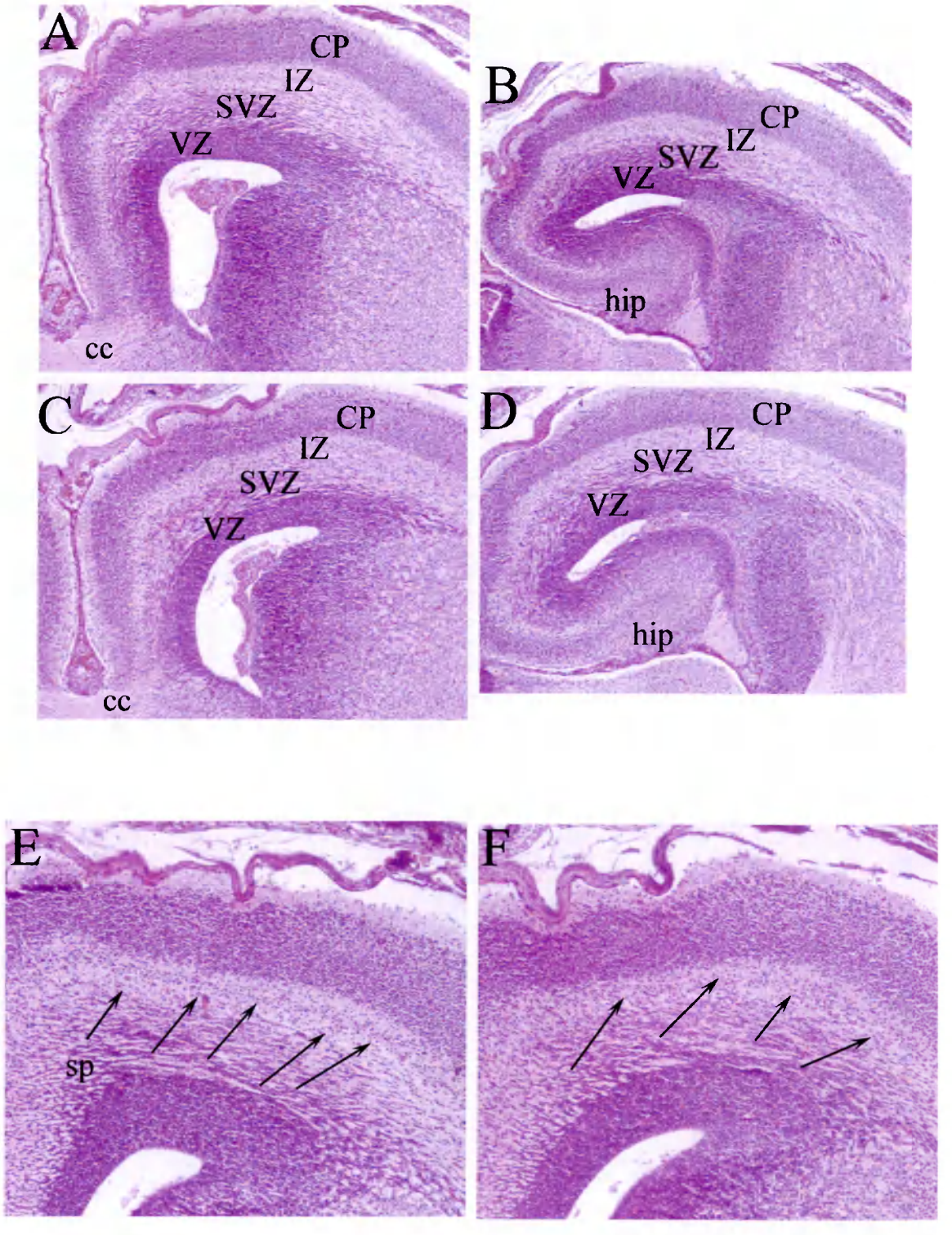


Figure 3.5

Hematoxilin-eosin stained frontal sections of E17.5 wild type (A, B and E) and knockout (C, D and F) embryos. Arrows in E and F point to the subplate just beneath the cortical plate (see text for details).

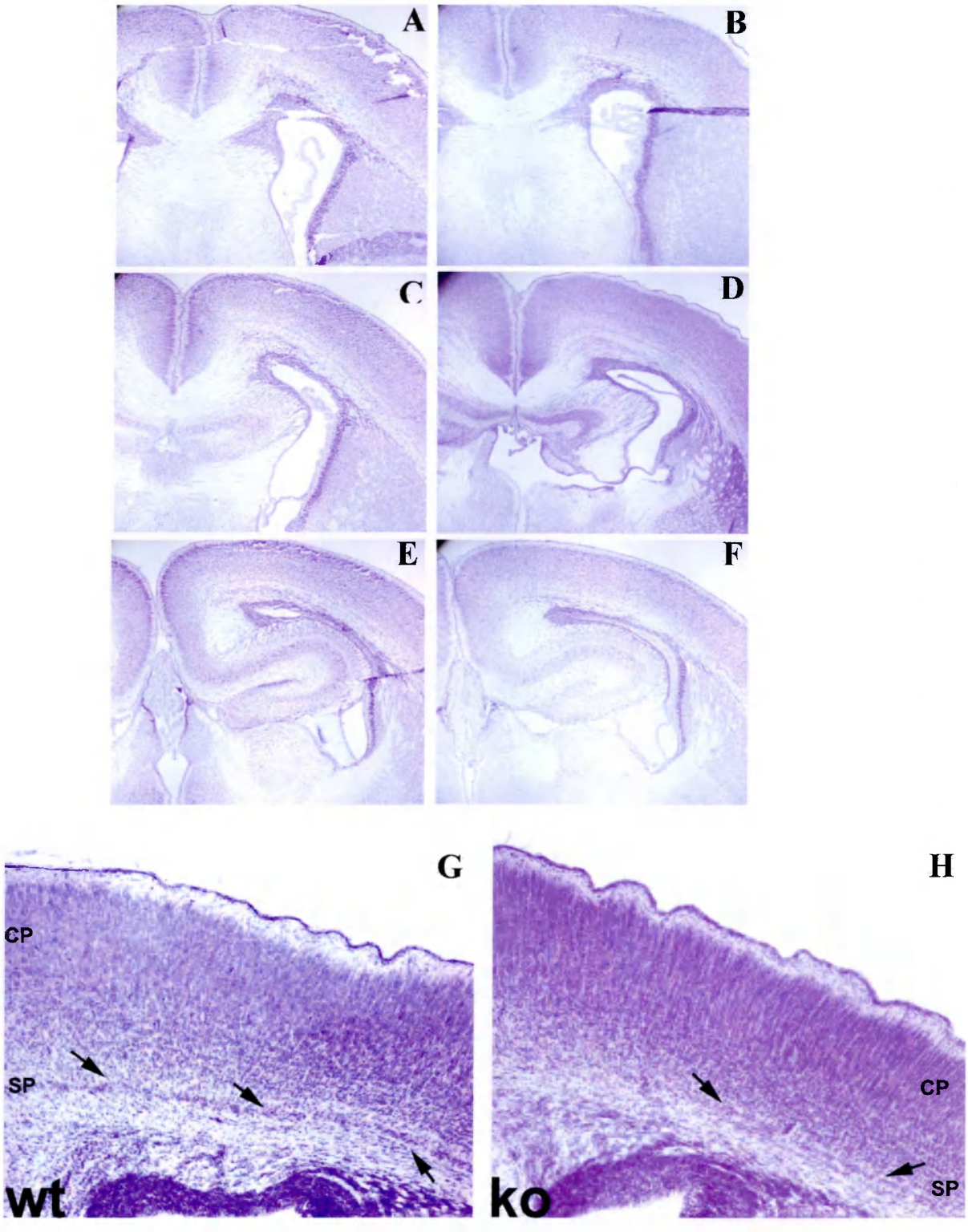


Figure 3.6

Frontal sections of P0 Emx1 null (B, D, F and H) and wild type (A, C, E, and G) brains stained with Nissl. (G, H) Highly magnified view of the medial versus lateral region of the cortex of a wild type and a mutant brain respectively; the subplate (arrows) in the mutant is hardly visible.

in the forming CP, and look at them singularly.

First, Emx2 immunocytochemistry (figure 3.7) allowed us to look at the VZ and the MZ in the developing mutant brain, and revealed that not only the $Emx1^{-/-}$ VZ was normal in thickness and cell density, but also Cajal-Retzius cells exhibited a normal phenotype in the mutant MZ (arrowheads in figure 3.7). These observations led us to hypothesise that in mutants the unaltered expression of Emx2, which has been implicated in directing processes of neuroblasts proliferation and subsequent migration, would ensure the normal proceeding of cortical neurogenesis.

To subsequently look at post-mitotic cells in the developing cerebral cortex we chose molecular markers specific of those cells.

GAP-43 is a protein linked to axon outgrowth during both development and differentiation. With the exception of the earliest developmental stages, at which this protein is also present at low levels in neuronal somata, the protein is present in axons and neuronal growth cone. We could not see any difference in the GAP-43 immunoreactivity when we look $Emx1^{-/-}$ cortices in comparison with the wild type ones (not shown).

Map2 (microtubule associated protein 2) is a microtubule-associated protein found exclusively in neuronal dendrites and somata. Map2 immunoreactivity can be detected in the cortical and subplate neurons of developing cerebral cortex. In $Emx1^{-/-}$ embryos, Map2 antibody stained neuronal cell bodies and processes in the forming cortical plate, whereas the ventricular zone was almost unstained. We did not detect any significant difference comparing those samples with the wild type littermates (figure3.8).

TuJ1, the neuronal specific class III tubulin, is one of the earliest markers expressed by post-mitotic neuronal cells. The antibody against TuJ1 stains layers of the forming cerebral cortex from the subventricular zone (SVZ) to the marginal zone (MZ) with different intensities; almost no reactivity can be found in the ventricular zone (VZ). In $Emx1^{-/-}$ cortices this pattern of expression was preserved (figure 3.9).

During cerebral cortex formation, radial glial bundles span from the ventricular to the marginal edge of the cortical wall since the very beginning of its

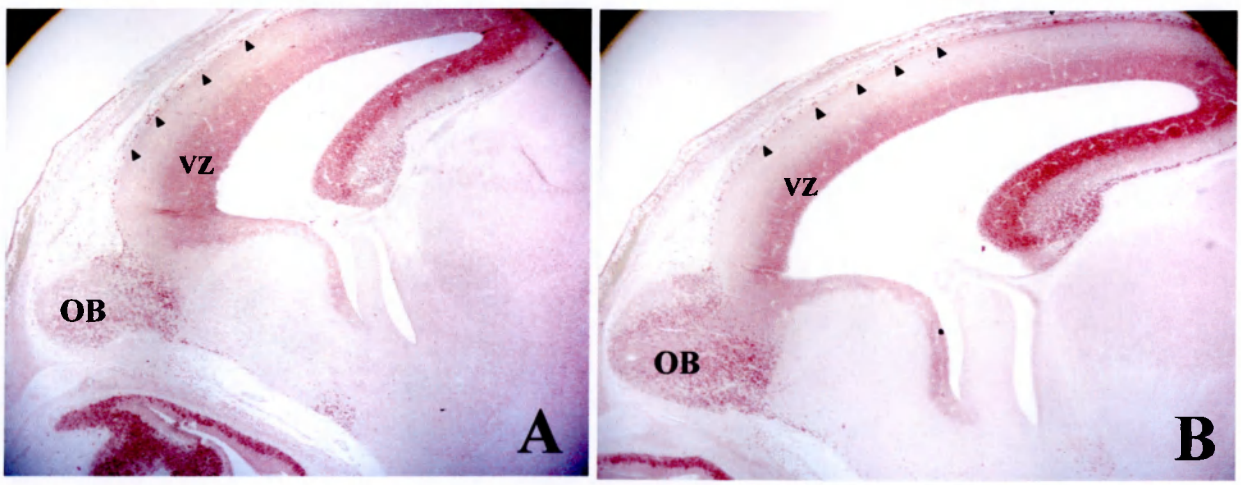


Figure 3.7

Analysis of EMX2 protein distribution in E15.5 sagittal sections of a wild type (A) and a mutant (B) brain. Emx2 expression in the mutant brain (B) is normally detected in the ventricular zone and in the Cajal-Retzius cells (arrowheads), and preserves the rostrocaudal gradient with a caudal maximum.

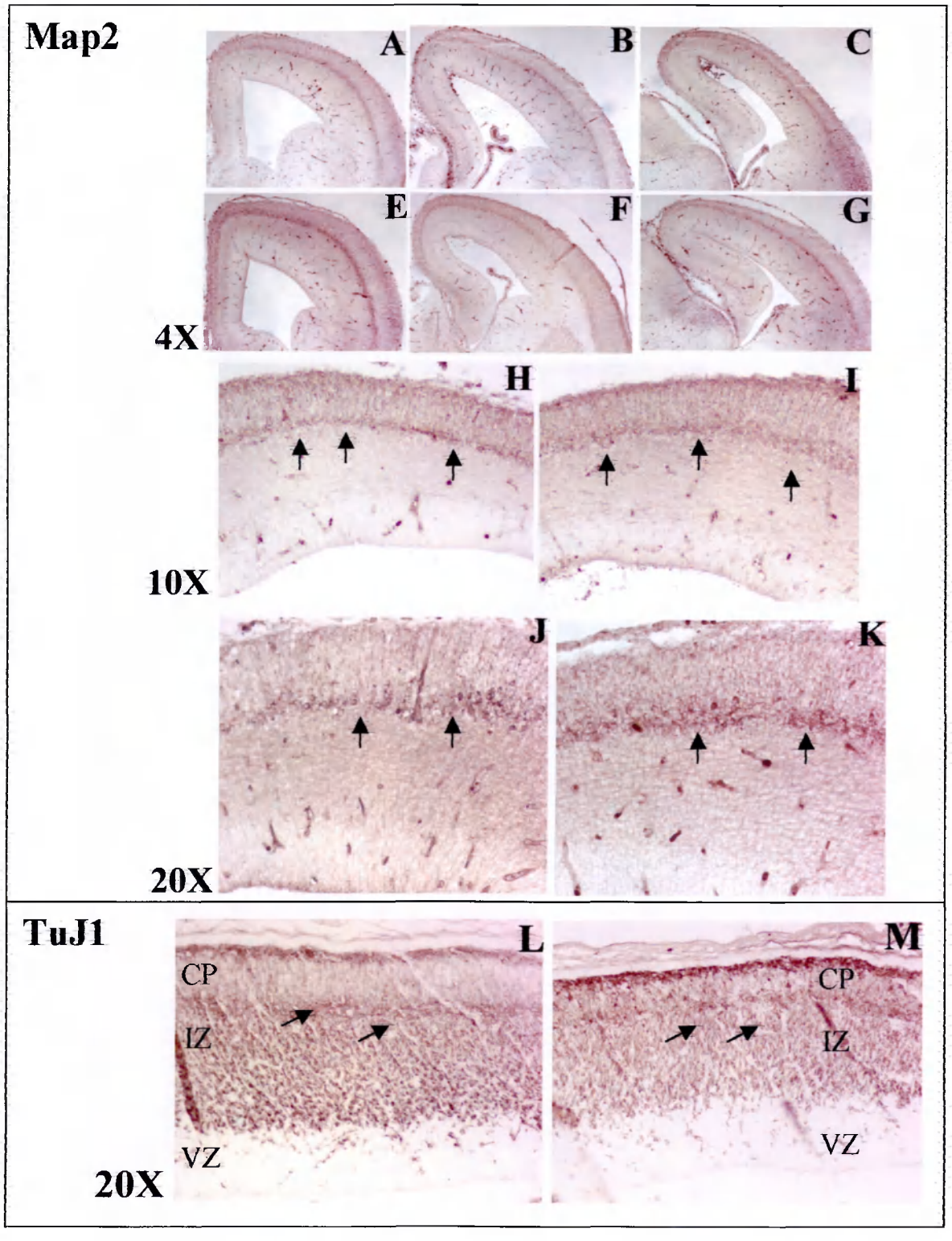


Figure 3.8

Map2 (A-K) and TuJ1 (L-M) immunocytochemistry performed on E15.5 frontal and sagittal sections respectively, from wild type (A, B, C, H, J and L) and mutant (D, E, F, G, I, K and M) embryos (see text for details). Arrows in H, I J, K, L and M point to the subplate.

development, and radially elongated neurons climb along the surface of these bundles during their translocation from the VZ to the forming CP.

In order to look at the radial glia anatomy in Emx1$^{-/-}$ cortices as well as in the wild type controls, we used antibody against GFAP and vimentin; however, we could not detect any difference between the two groups of samples at all stages of the embryonic development analysed (not shown). As a confirmation, not only TuJ1 and Map2 immunocytochemistry demonstrated that in Emx1 mutants neurons seem to normally follow the neurogenetic program that leads to the formation of a normal cortex, but also BrdU pulse-labelling experiments (see below) revealed that the radial distribution of post-mitotic cortical neurons is not affected by the deletion of Emx1 gene.

This analysis suggests that the molecular and anatomical changes associated with the development of the neocortex, and culminating in a very precise sequentially formed layers of neurons, were not perturbed by the Emx1 gene inactivation.

On the other hand, we found that the subplate in the mutant cortex was sometimes poorly differentiated. In order to directly look at the subplate cells, immunocytochemistry with the antibody against calretinin (CR), which is a specific marker of this transient cortical layer (Fonseca et al., 1995), was used. The expression of CR during development was diminished in the mutants; however, the defect was more evident in the medial part of the cortical wall (figure 3.9). Moreover, CR is also specifically expressed by Cajal Retzius cells in the marginal zone; but in this layer we could not detect any defect.

Finally, we studied the expression of Tbr1 in Emx1 deficient mice.

Tbr1 is a member of the *brachyury* family of T-box transcription factors, which has been shown to orchestrate cortical development. To date, Tbr1 knockout mice exhibit disruption of cortical lamination and axonal connections.

In Emx1 mutants, this gene is normally expressed. In fact, it is only found in postmitotic neurons, first in the preplate as soon as it is formed and later in its derivatives (MZ and subplate), and subsequently in the CP and IZ (figure 3.10, A). As cortical lamination differentiates around birth, Tbr1 expression is evident in all cortical layers but most prominently in layer 2/3 and 6, and in the subplate (Figure 3.10, B). Moreover, we cannot detect alteration in the Tbr1 subplate localization.

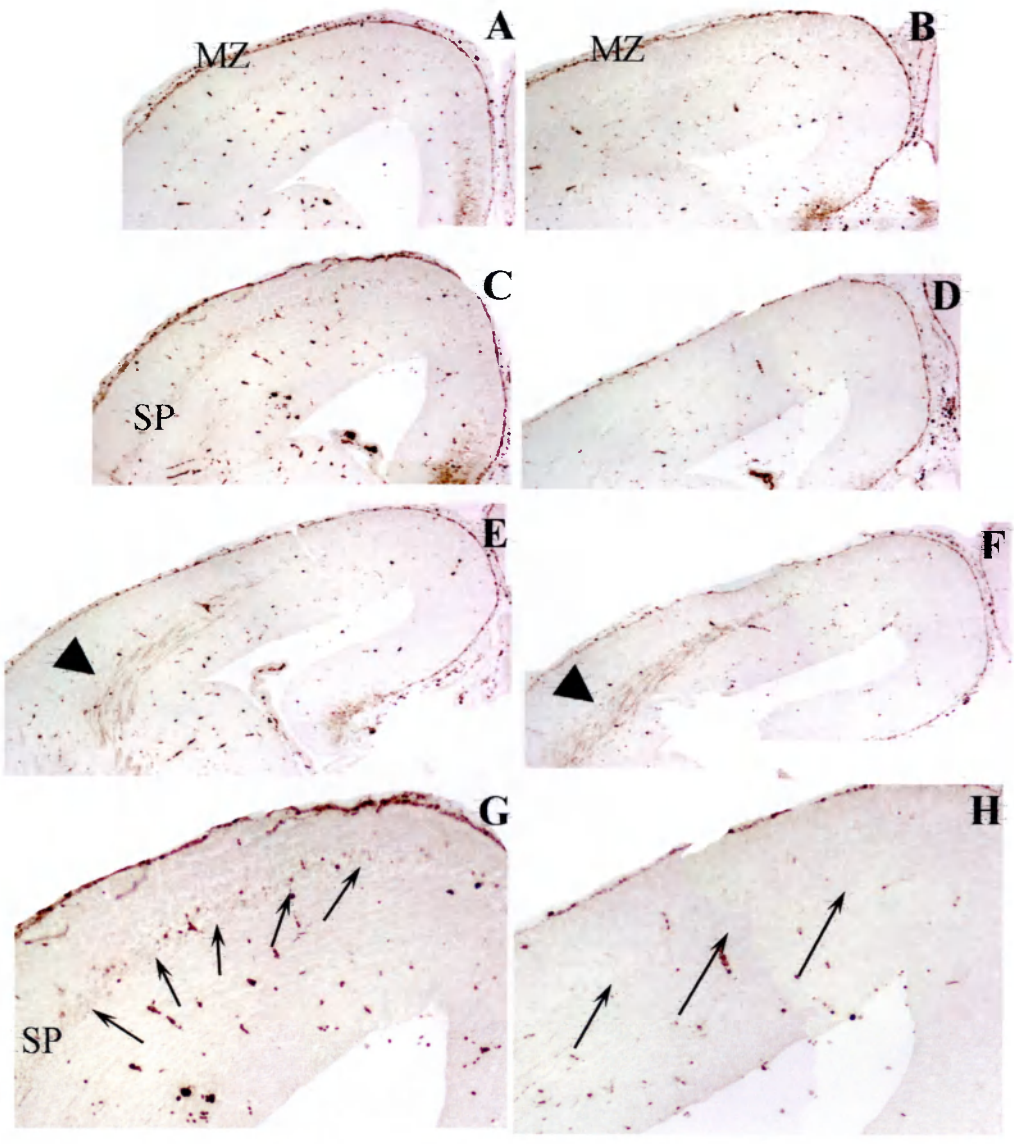


Figure 3.9

Calretinin immunocytochemistry on frontal section of E15.5 wild type (A, C, E, G) and mutant (B, D, F, H) brains. The immunoreactivity is detected at the level of subplate (arrows in G) and MZ; but in the mutant it is absent from the medial portion of the subplate (arrows in H). In caudal sections, the antibody stains incoming axons from the thalamus (arrowheads in E and F).

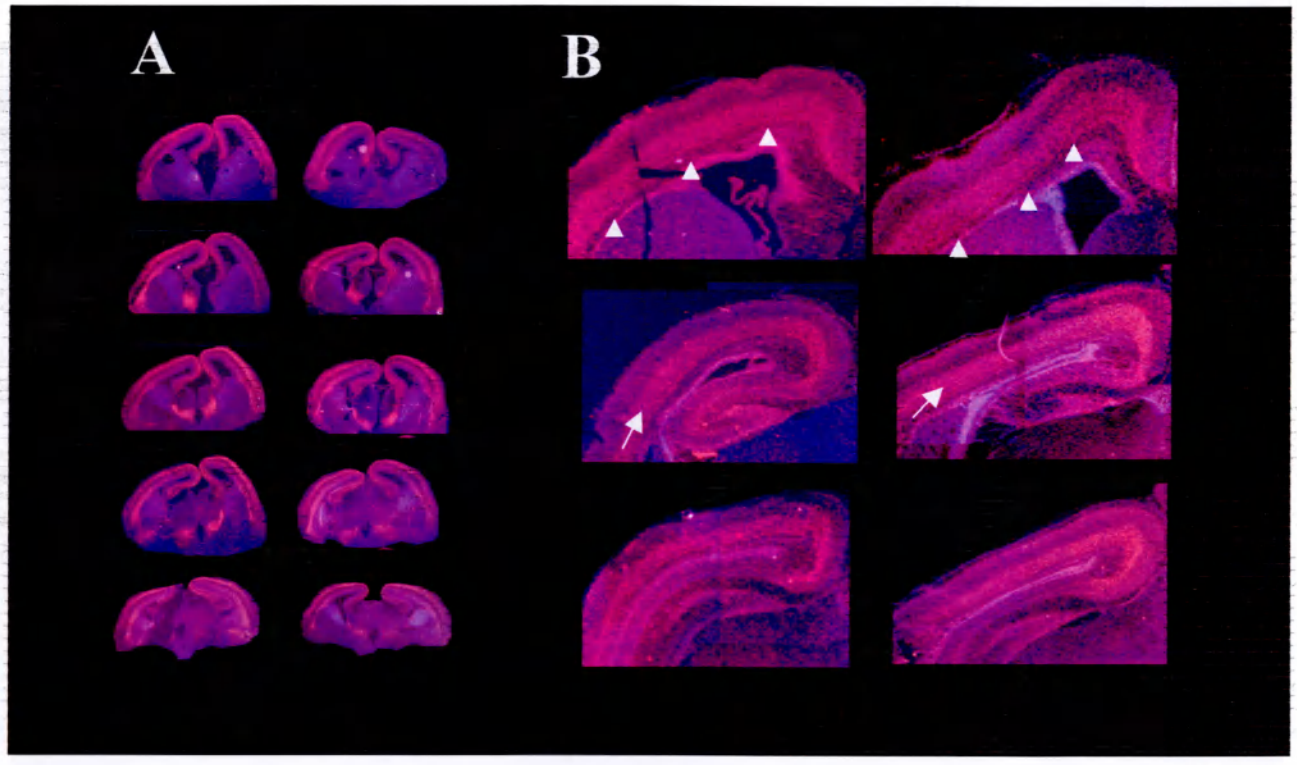


Figure 3.10

Tbr1 expression in E15.5 (A) and P0 (B) wild type (left panels) and Emx1 deficient (right panels) frontal brain sections. (A) At E15.5 high expression of Tbr1 is visible in the marginal zone, cortical plate and subplate both in the Emx1 mutant (right) and wild type (left) embryos; no expression is detected in the ventricular zone. (B) At P0 the expression is strong in layer 6 (white arrows) and subplate (arrowheads), whereas cells in other cortical layers express Tbr1 at lower levels. No difference is found comparing the wild type (left) with the mutant (right) brain.

3.2.3 Proliferation of cells in the neocortex of Emx1 deficient mice

To systematically compare either the rate of proliferation and the migratory behavior of cells fated to form the neocortex in Emx1 null versus Emx1 wild type embryos, we pulse-labelled neurons born at E11.5, E12.5, E13.5, E15.5, E17.5 by BrdU. Embryos (for each genotype and each labelling, four animals were analysed) were harvested after 90' in a first set of experiments and at P0 in a second one. Telencephalons were frontally sectioned, and the total number and the distribution of BrdU labelled cells were scored in neocortical sectors at three standard rostro-caudal levels. In particular, to investigate how cortical neuroblasts distribute into the forming cortical plate, at each level, the neocortical sector was partitioned in bins of equal radial width (200μm). The number of bins was always the same for mutants and the wild type littermates, indicating that the thickness of the cortical wall was almost similar. The bins were numbered from ventricular to marginal, and the number of BrdU positive cells in each bin was counted.

However, this analysis did not reveal any abnormality in the BrdU uptake, and therefore in the rate of cell proliferation and subsequent radial distribution in the forming cortical plate of Emx1 null embryos.

Here, I will show some representative examples of the results obtained from this study.

In figure 3.11 A, C, E, and B, D, F are frontal sections from E11.5 wild type and mutant embryos respectively, taken at the three standard levels along the rostrocaudal axis and stained with the antibody against BrdU. Observation of this staining did not allow detecting any difference between the wild type and the Emx1 null telencephalons. Graphs in figure 3.11 show the mean values (± S.E.M.) of the total number of BrdU positive cells in the wild type and the mutant brains at the three rostro-caudal levels.

We could not detect any significant difference in the rate of proliferation of cortical neuroblasts between the Emx1 mutant and wild type littermates.

The same kind of analysis has been performed at later stages of embryonic development (figure 3.12).

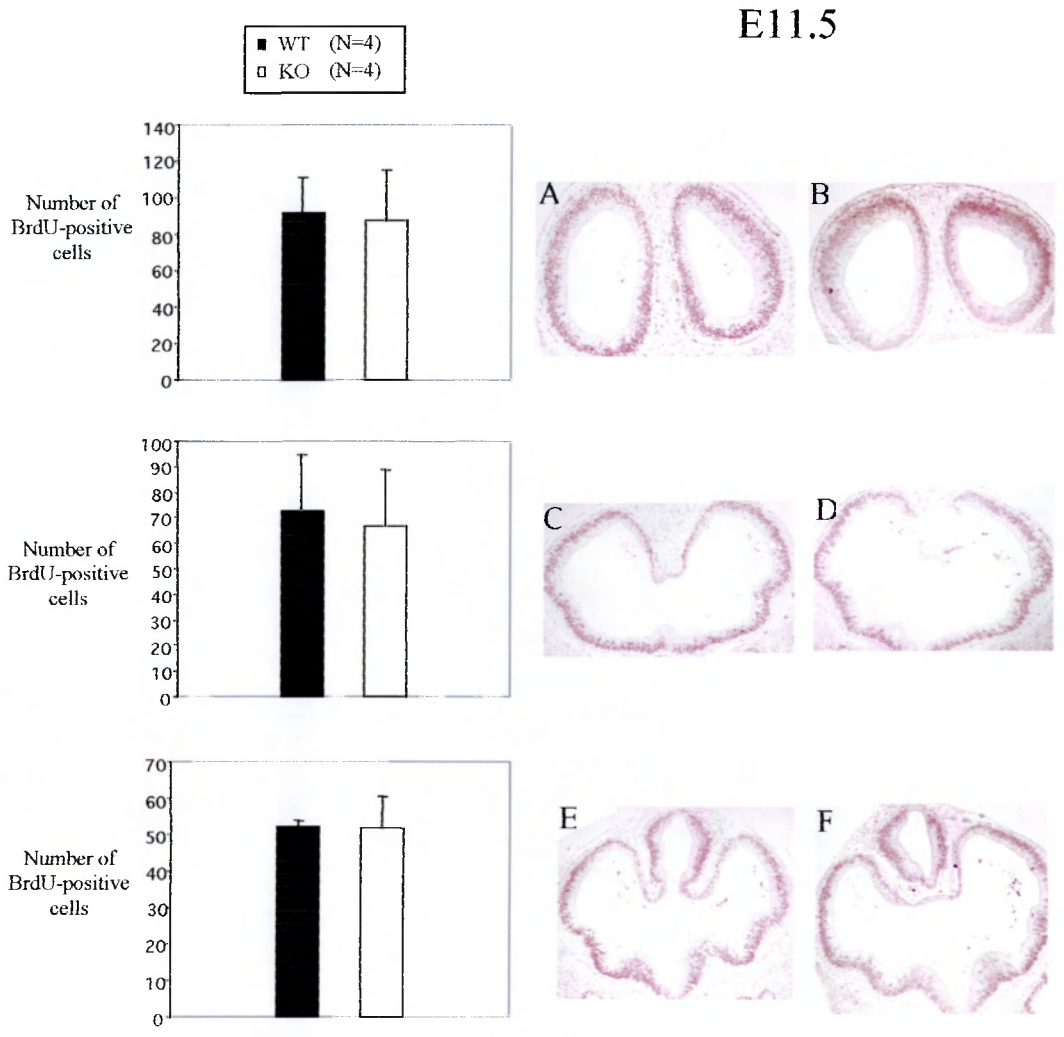


Figure 3.11

BrdU immunocytochemistry on frontal sections of E11.5 mutant (B, D and F) and a wild type (A, C and E) embryos that were pulse-labelled with BrdU. Pregnant animals were injected with BrdU (100μg/g) 90' before they were killed Embryonic brains were processed for immunocytological detection of BrdU as described in Chapter II. BrdU labelling does not reveal differences comparing wild type (A, C, and E) sections with the mutant (B, D and F) ones. The total number of BrdU-positive cells in a standardised sector of the cortical wall, was quantified and shown graphically, at each level along the rostrocaudal axis. No significant difference in the proportion of BrdU-positive cells was observed in cortical sections of mutant embryos compared with the wild type ones.

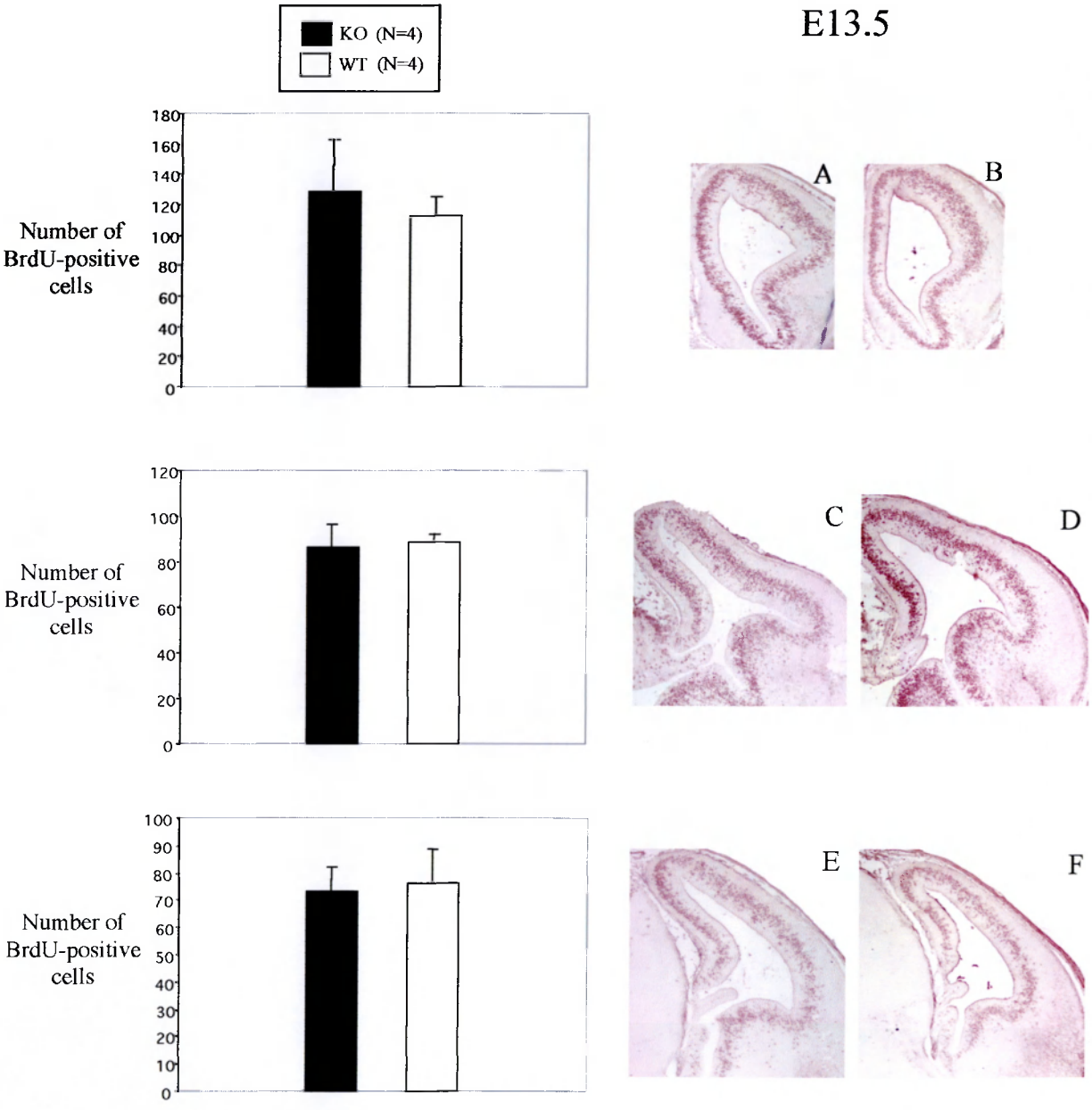


Figure 3.12

BrdU pulse labelling reveals a comparable rate of proliferation in E13.5 Emx1 mutant (B, D, and F) and wild type A, C and E) cortex (for further details see text and figure 3.11). (N, number of samples analysed for each genotype).

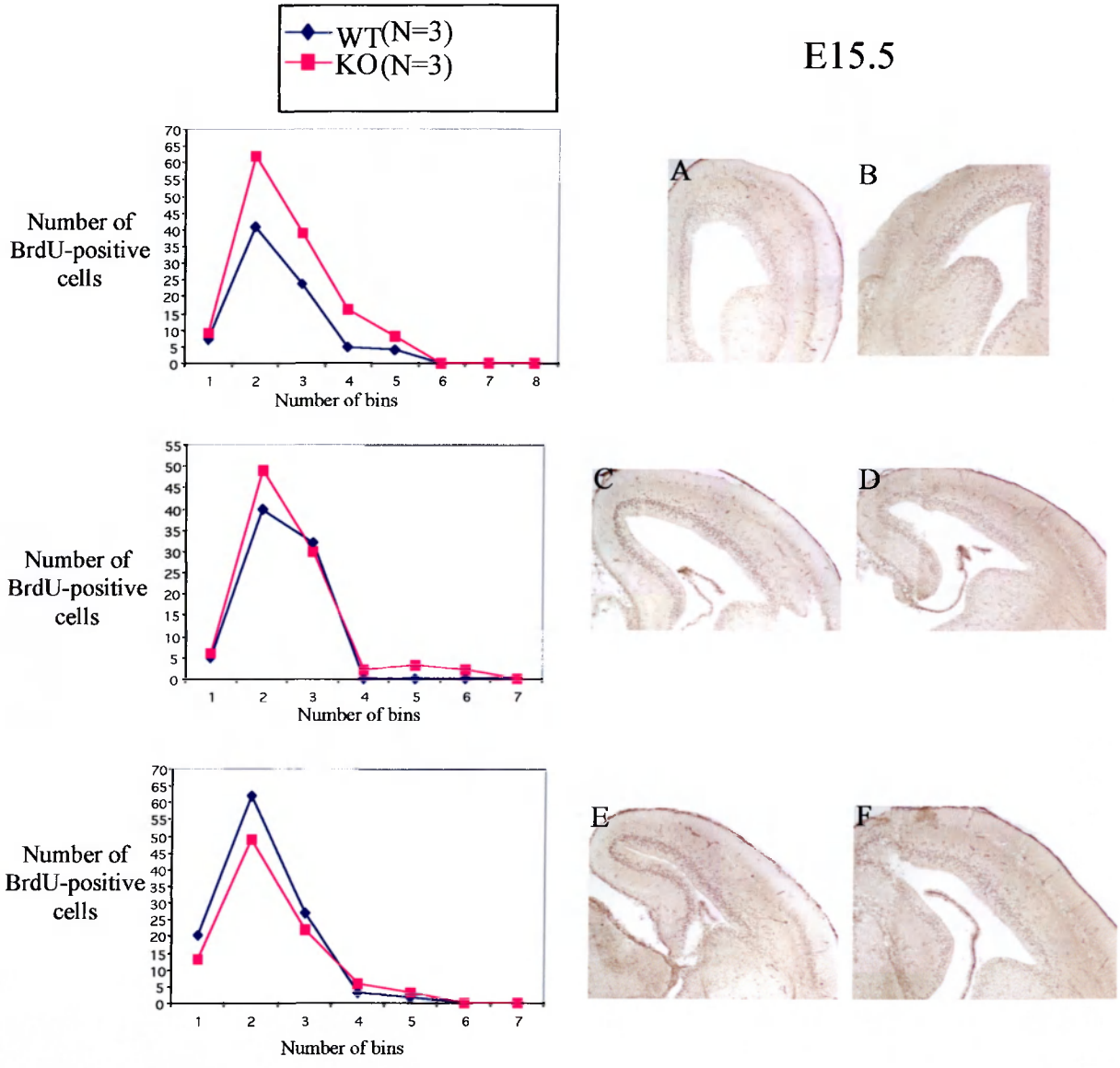


Figure 3.13

Radial distribution of E15.5 BrdU pulse-labelled cells in the neocortex of wild type and Emx1$^{-/-}$ embryos. (A-F) Photographic examples of BrdU-immunostained frontal sections of E15.5 wild type (A, C, and E) and mutant (B, D, and F) mouse embryos. Embryos were pulse-labelled at E15.5 and harvested after 90'. Graphics synthesize the total number and radial distribution of BrdU positive cells in a standardised neocortical sector (for details, see text); the radial distribution of BrdU-positive cells at three rostrocaudal levels was obtained by plotting the average of the total number of labelled cells located in each bin against the bin number. The peaks in these graphs indicate that the vast majority of cells are located in the ventricular and subventricular zone after 90' from the BrdU injection; nevertheless, a portion of BrdU-positive cells have started to migrate toward the CP and localise in the intermediate zone. No differences are found in the number and radial distribution of BrdU-labelled cells between wild type and mutant embryos. (N, number of samples analysed)

The comparison between the total number as well as the radial distribution of BrdU labelled cells in E15.5 mutant and normal brains, is shown in figure 3.13.

Finally, the radial distribution of BrdU-pulse labelled cells was also found not to be altered in the neocortex of P0 $Emx1^{-/-}$ mice that have been injecting with BrdU at embryonic stages E12.5, E15.5, E 17.5 (not shown).

The results of this study confirmed that in the absence of a functional Emx1 gene, cortical neuroblasts normally proliferate and migrate, forming a normal cortical plate.

3.3 EXPRESSION PROFILE OF Emx2 GENE IN THE DEVELOPING $Emx1^{-/-}$ FOREBRAIN

In order elucidate the reason for the absence of obvious brain development abnormalities in Emx1 knockout mice, we hypothesised that the loss of Emx1 functions has been compensated for by other genes, especially those having an overlapping expression pattern. In this regard, it is reasonable to assume that the other member of the *Emx* gene family, Emx2 could play such a role.

Emx2 has a partially overlapping expression pattern to that of Emx1. In particular these genes are expressed in a nested pattern in a time interval corresponding to major events in cortical neurogenesis. This not only suggests a possible role for Emx genes in determining the identity of developing cortex, but also does not exclude a possible redundancy and interchangeability in some of their functions.

Therefore, we decided to look at the expression of Emx2 gene in the developing $Emx1^{-/-}$ brains, utilising both anti-Emx2 immunocytochemistry and in situ hybridization analysis. However, we could not detect any alteration of Emx2 expression profile in $Emx1^{-/-}$ developing cortex in comparison with the wild type one. (figure 3.14).

In fact, in $Emx1^{-/-}$ embryonic cortices, both Emx2 transcript and protein were detected in the MZ and in the VZ, where the expression followed the normal gradient with a medial-caudal maximum. Nevertheless, those observations do not exclude that Emx2 is still involved in rescuing the absence of Emx1. The best experimental

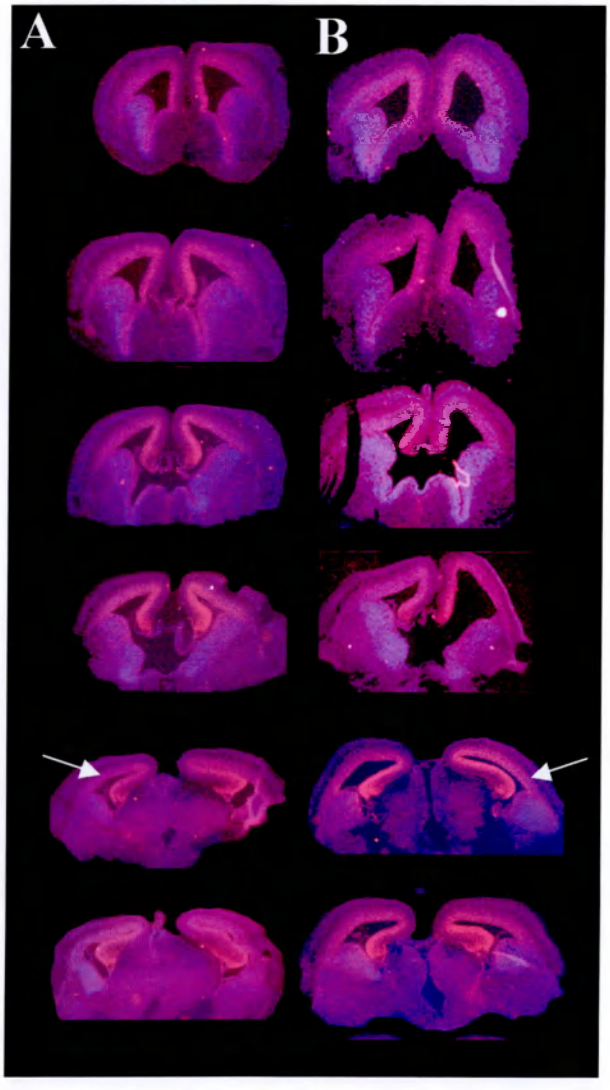


Figure 3.14

Distribution of Emx2 transcript in rostral versus caudal frontal sections of E15.5 wild type (panel A) and Emx1$^{-/-}$ (panel B) telencephalons. In both cases, Emx2 transcript is detectable in the ventricular zone of the dorsal telencephalon, according to the rostral versus caudal and lateral versus medial gradient of expression (arrows indicate point to the decreasing intensity of the signal in the lateral part of the telecephalon).

strategy to study the possible redundancy between the two Emx genes will be to look at the double knockout mouse.

3.4 Emx1 GENE EXPRESSION FROM THE EMBRYOGENESIS TO THE ADULTHOOD

The expression pattern of Emx1 was already known (Simeone et al., 1992; Gulisano et al., 1996; Briata et al., 1996), when we started our study of the phenotype of Emx1 loss of function mouse. Nevertheless, we were particularly interested in assessing if the expression of this gene in the developing cerebral cortex was graded. Therefore, we decided to repeat the expression analysis; and to this purpose, we used wild type (C57BL/6) animals to look at the distribution of Emx1 mRNA and protein during both embryogenesis and post-natal life.

3.4.1 Recapitulation of the Emx1 transcript temporal and spatial distribution in the developing and post-natal forebrain

We have performed in situ hybridization analysis on wild type C57BL/6 embryos at different embryonic stages and post-natal mice.

We found that during development, the Emx1 transcript distribution is graded along both the anterior-posterior and the lateral-medial axes with a posterior-medial maximum, resembling the expression profile seen for Emx2 transcript at same embryonic ages (figure 3.15 and 3.16).

At all embryonic and post-natal stages analysed, we found that Emx1 transcript is present across the entire thickness of the cerebral wall. We confirmed the previously reported graded expression in postnatal forebrain where stronger expression of Emx1 is detectable in more posterior regions and in particular in the archicortex; moreover we found that these anterior-posterior differences are transient as previously Gulisano et al. (1996) have reported.

Around P20, it not possible to detect any difference in the intensity of the signal along the lateral-medial axis (figure 3.17).

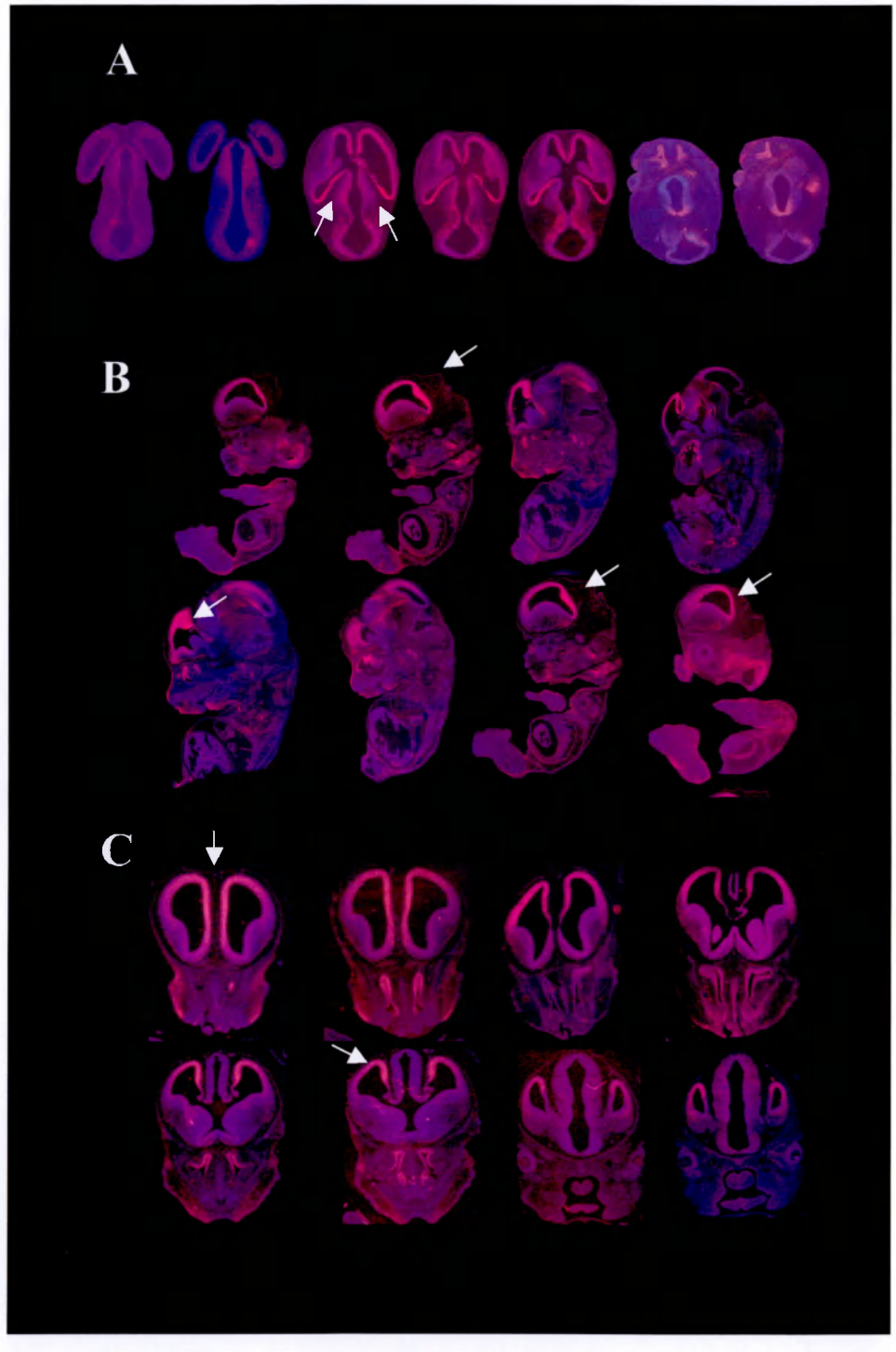


Figure 3.15

Emx1 expression in E12.5 coronal (A), sagittal (B) and frontal (C) sections of mouse embryos. Arrows in panel A point to the medial-caudal Emx1 hybridization signal. In the sagittal plane (panel B) the anterior versus posterior gradient is visualized, whereas in panel C, the medial expression of the gene can be easily detected.

3.4.2 EMX1 protein in the olfactory system

The EMX1 protein distribution in the developing and post-natal forebrain of mouse has been studied in detail by Briata et al. (1996).

Those authors have shown that the protein distribution essentially overlaps the expression domain of the Emx1 transcript (Gulisano et al., 1996).

We performed immunocytochemistry with the same polyclonal antibody utilised by Briata et al. (1996), that specifically recognises EMX1 protein (a gift from G.Corte). Using sections from wild type E12.5, E15.5 and E17.5 embryos, we confirmed that the protein is present in cell of the developing cerebral cortex as well as in the olfactory bulbs and hippocampus throughout embryogenesis (figure 3.18). We also found the presence of the protein in the nuclei of cortical cells and hippocampal formation of post-natal (P15) brains (data not shown).

However, Briata et al. (1996) reported that EMX1 protein is present along the entire length of the developing olfactory nerve in late gestation and post-natal mice. In particular, their immunocytochemistry analysis showed that, while in cells of the dorsal telencephalon EMX1 localises in the nuclei, by mid-gestation until several days after birth, the protein is also detectable in axons of neurons of the developing olfactory nerve.

This finding raised at that time several questions, first about the role that this protein, believed to act as transcription factor, might play along axons, and secondly, about the mechanisms by which the protein becomes localised in axons, if it is translated in situ or it is transported from the nucleus

We decided to look at the presence EMX1 protein in the olfactory nerve. To this purpose, we performed immunocytochemistry with the anti-Emx1 antibody to stain both coronal and sagittal sections from E15.5, E17.5 and P15 $Emx1^{-/-}$ brains and wild type littermates.

As expected, we could not detect any immunocytochemical signal in the cortex of Emx1 null mice as well as in any other brain localisation (figure 3.18). However, we obtained the same strong anti-Emx1 staining in the olfactory nerve of Emx1 null mice (figure 3.18).

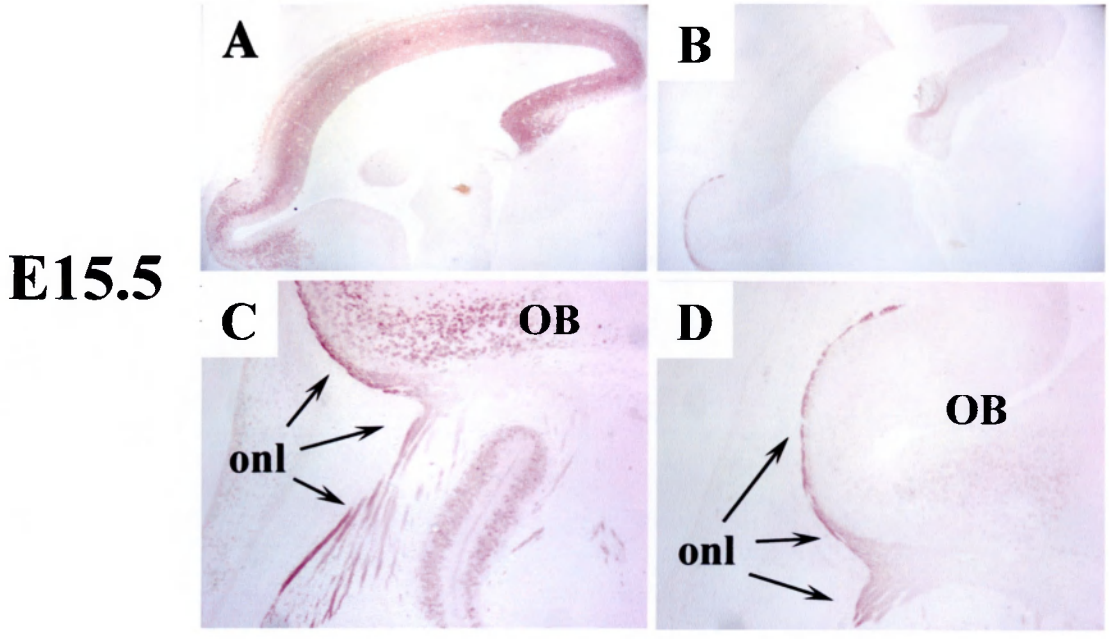


Figure 3.18

EMX1 immunocytochemistry on E15.5 sagittal sections of wild type (A, C) embryo and Emx1 null (B, D) embryos. EMX1 is found in all cortical layers with the exception of layer 1 (A) and in cells of the olfactory bulb. As expected the protein is not present in the mutant cortex (B). The antibody also stains the olfactory nerve layer (onl) along its entire length from the olfactory epithelium to the olfactory bulbs (C). However, in the mutant a similar signal is detected (D) suggesting that the antibody in this location recognises one or even more proteins, that obviously cannot be EMX1.

This result demonstrated that the antibody against Emx1 protein is able to recognise one or even more other proteins specifically expressed in the olfactory nerve that cannot be EMX1.

Therefore, the previously described presence of EMX1 protein in the olfactory nerve has to be considered as an artefact due to the aspecificity of the antibody in this location.

3.5 SPECIFICATION OF CORTICAL AREA IDENTITIES IN THE ABSENCE OF A FUNCTIONAL Emx1 GENE

Regionalization of the cerebral cortex has been proposed to involve two phases: an early arealization phase, which does not require thalamic inputs and is regulated by intrinsic factors, and a later refinement under the influence of thalamic projections. Recently a number of intrinsic factors have been identified, in particular Emx2, Pax6 and COUP-TF1.

The spatial and temporal expression pattern of Emx1 suggests a potential role of this gene in specification of neocortex and in maintaining cortical identity.

To determine if Emx1 was a regulatory factor for regionalization of the cerebral cortex, we investigated the region-specific expression of marker genes including: Cad6, Id3, Id2, Lamp, COUP-TF1, and Emx2.

Cadherin6 (Cad6) belongs to a family of cell adhesion molecule that are important for morphogenesis of the CNS. Cad6 mRNA is detectable in the basal telencephalon as well as in the cerebral cortex. In particular, the rostral and intermediate pallium, Cad6 expression spans both sides of the cortico-striatal notch, in the neocortex and in the paleocortex. We found that in Emx1 mutant brains, the Cad6 expression domain does not display any distortion compared to that of wild type littermates (figure 3.19).

The helix-loop-helix transcription factor Inhibitor of differentiation 2 (Id2), is expressed in a region- and lamina-specific manner. Its expression is detected in the subplate and layers 6, 5, and 2/3, at different levels according to the rostro-caudal location (Bulfone et al., 1995). In Emx1 mutants the expression profile of Id2 was found unaltered (not shown).

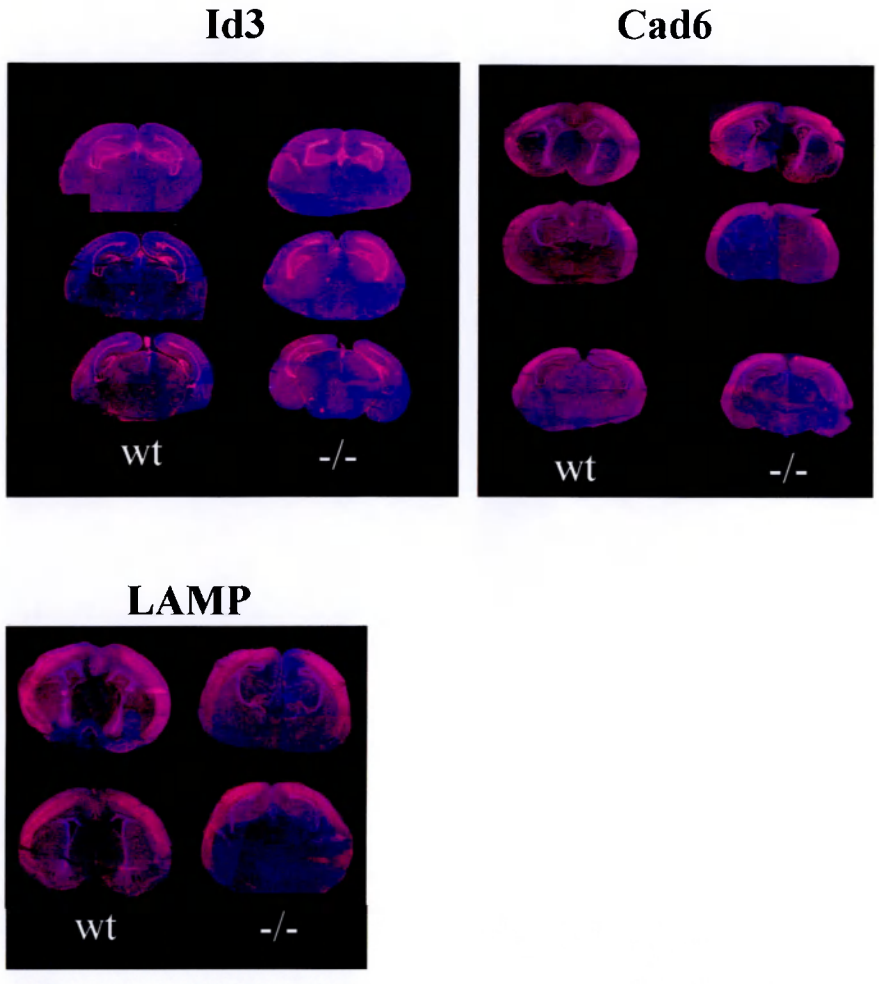


Figure 3.19

Expression patterns of Cad6, Id3 and LAMP in normal (wt) and Emx1$^{-/-}$ cerebral cortices. For each gene and each genotype a set of frontal section at E19.0 is shown.

Another helix-loop-helix transcription factor Inhibitor of differentiation 3 (Id3) is specifically expressed in the medial cortical wall, where it localises to the cingulate cortex and the archicortex. An unaltered expression pattern was diplayed by Id3 in Emx1 mutant brains. Figure 3.19 shows an example the comparison between Id3 mRNA distribution in E19.0 wild type and Emx1 null brains.

The limbic system-associated membrane protein gene (lamp) is normally expressed in the basal telecephalon as well as in the lateral telecephalon. Its distribution in mutant brains was practically identical to that detected in the wild type brains (figure 3.19).

Emx2 has been recently shown to be essential, together with Pax6, for specification of cortical areas. In particular, Emx2 and Pax6 seem to regulate regional expression of genes responsible for area identity (Bishop et al., 200). Our analysis of Emx2 expression in $Emx1^{-/-}$ embryos did not reveal any change in the distribution of both transcript and protein.

COUP-TF1 is an orphan member of the nuclear receptor superfamily that has been shown to have a role in specification of the neocortex and in maintaining cortical identity. In fact, mice with a null mutation of COUP-TF1 gene exhibit altered expression of many marker genes in the cortex as well as miswired area-specific connections between the cortex and the thalamus (Zhou et al., 2001). Starting from E11.5 COUP-TF1 expression has a graded expression profile in the neocortex with caudal-lateral maximum. This expression gradient in maintained in the cortical plate even after birth, and has also been by differential display PCR analysis (Liu et al., 2000). We found that COUP-TF1 expression is not altered in its cortical localisation and gradient along the rostral-caudal and the lateral-medial axes in Emx1-/- embryos. However we have found that the level of expression is increased in those locations, especially in the archicortex of P0 brains (figure 3.20).

It has been proposed that COUP-TF1 functions independently from Emx2 and Pax6 in regulating neocortical regionalisation; moreover, Emx2 and Pax6 would act in promoting and initiating the early regionalisation, COUP-TF1 would have a role in maintaining this regional identity.

These data together suggest that area identities in Emx1 deficient cortex are encoded without disturbances. It is therefore possible that other genes, implicated in

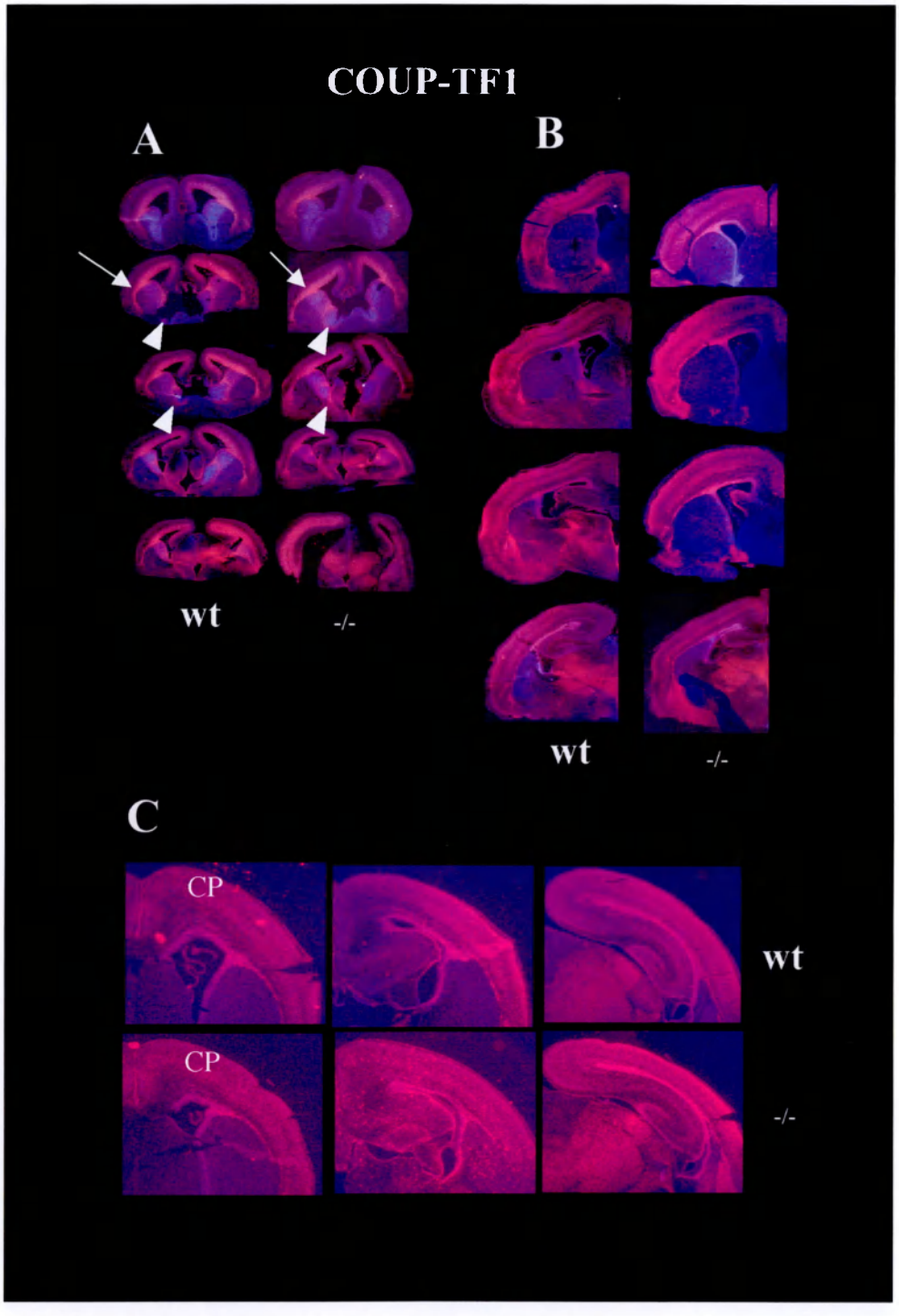


Figure 3.19

COUP-TF1 expression in E15.5 (A) and P0 (B, C) frontal sections of normal (wt) and Emx1$^{-/-}$ brains. A, COUP-TF1 showed a graded pattern in the neocortex with high-lateral to low-medial expression in frontal view, that is preserved in the knockout. In particular, the gene is expressed predominantly in the neocortical ventricular zone and in the ganglionic eminence (arrowheads). Around birth (B, C high magnification), COUP-TF1 is expressed higher in the CP than in the VZ, and the caudolateral gradient is maintained. However, in the mutant brain the signal seems to be stronger, even maintaining the normal spatial location.

the regulation of such processes, might compensate for the absence of Emx1. One of these genes could be Emx2, whose homology with Emx1 and similar expression profile make it an excellent candidate as redundant or compensating factor; another could be COUP-TF1, which is overexpressed in the absence of Emx1 and has been recently shown to be involved in regulation early cortical arealization.

3.6 ESTABLISHMENT OF NORMAL CORTICAL AND THALAMIC PROJECTIONS IN Emx1 KNOCKOUT MICE

We investigated if the absence of a functional Emx1 gene affects the establishment of the specific axonal projections between the cerebral cortex and the dorsal thalamus, during embryonic development.

This analysis would have addressed two main questions. The first was related to the specification of cortical area identities. In fact, the arealization processes at late gestation have been shown to be partially dependent on the arrival of thalamic axons into the neocortex. The second point was related to the subplate defects observed in Emx1 deficient cortices.

It has been suggested that subplate cells are implicated in guiding thalamocortical afferent axons into the neocortex and cortical efferent axons to their subcortical targets (McConnell et al., 1989, Ghosh et al., 1990; De Carlos and O'Leary 1992; Ghosh and Shatz, 1993). We therefore wanted to elucidate if the subplate defects observed in a percentage of knockout mice would be reflected in misrouting of afferent and efferent projections.

We chose two experimental strategies. We first injected carbocyanine dye (DiI) to follow corticofugal and thalamocortical pathways; and subsequently, we confirmed the results by immunocytochemistry with antibodies against the neural adhesion molecules TAG-1 and L1 to mark cortical and thalamic axons respectively.

3.6.1 DiI Tracing

To directly examine corticothalamic and thalamocortical axons, we placed DiI crystals into the cortex (usually the presumptive somatosensory region) and the dorsal thalamus at all embryonic stages from E14.5 to E18.5. We usually visualized both projection systems in the same brain by injecting a crystal in the cortex of one

hemisphere to label corticothalamic axons, and anther crystal into the dorsal thalamus of the opposite hemisphere to label the thalamocortical ones.

Placement of DiI crystals into the cortex of $Emx1^{-/-}$ embryos at E14.5 showed that cortical axons grow normally toward the subcortical telecephalon (sub-pallium), where they meet the axons coming from the thalamus (figure 3.21). Here the two projection systems remain in close interaction for not longer than one day. It has been hypothesised that after reaching the subpallium, these projection systems exchange guidance cues necessary for completing their trajectories: this type of mechanism ensures that thalamocortical and corticothalamic axons project reciprocally (Hevner et al., 2002) (see scheme in figure 3.21, A). We found that Emx1 gene inactivation does not alter this mechanism. By E15.5, in fact, in $Emx1^{-/-}$ brains cortical projections have overcome the internal capsule and are directed to the thalamus; they normally reach the thalamus by E16.5. In fact by E17.5 (figure 3.21, B) the $Emx1^{-/-}$ brain shows a comparable anterograde and retrograde labelling of the cortex as well as of the thalamus.

The results of this study indicate that Emx1 is not necessary for the cortical and thalamic axons appropriate projection to reciprocal targets.

Moreover, previous studies have shown that Emx1 is strongly expressed by subplate neurons. Those neurons have been shown to provide a scaffold for the growth of thalamocortical connections (McConnell et al., 1989).

It was conceivable that Emx1 would directly have provided a local guidance cue for thalamic axons innervating the cortex.

However, our results show that in Emx1 mutant brain mutation the formation and the trajectories of both growing corticothalamic and thalamocortical projections are not affected.

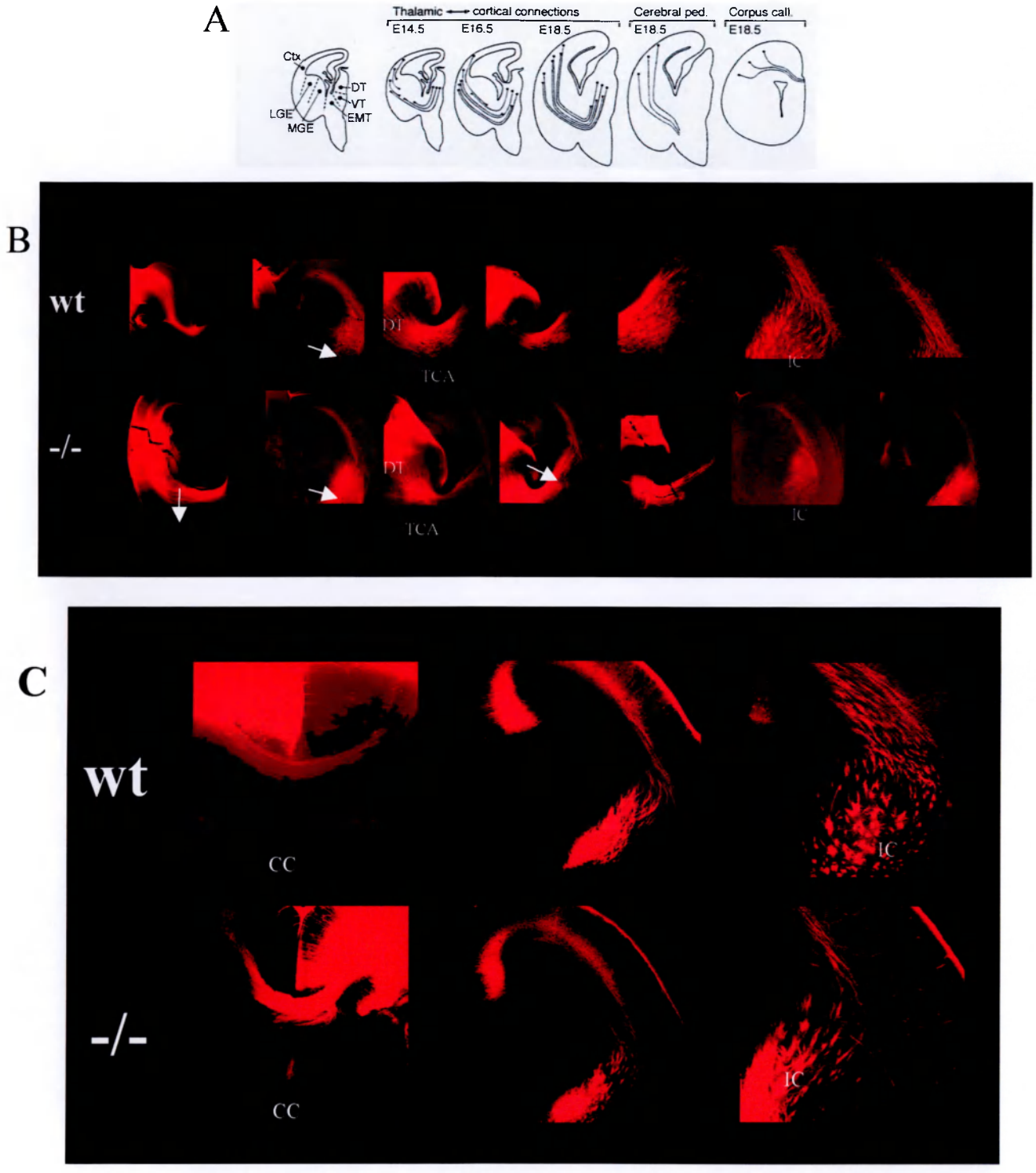


Figure 3.21

Summary of axon pathfinding results. Panel A represent a scheme of the formation of axonal projection between the cortex and the subcortical regions throughout embryogenesis. In B and C there examples of axonal projections both anterogradly and retrogradly traced with DiI, in E17.5 and E19.0 frontal sections respectively, of normal (wt) and Emx1$^{-/-}$ brains. Arrows point to the place where the two projection systems meet each other. CC, corpus callosum; IC, internal capsule; TCA, thalamocortical axons; DT, dorsal thalamus.

3.6.2 TAG-1 and L1 immunocytochemistry

The development of projection systems in the forebrain has also been studied using immunocytochemistry with antibodies against the neural adhesion molecules TAG-1 and L1 to mark cortical and thalamic axons respectively.

TAG-1 expression pattern was seen unaltered in $Emx1^{-/-}$ mice (figure 3.22, A). This molecule is first detectable at E12.5 in the primordial plexiform layer of the developing cerebral cortex (Denaxa et al., 2001). Two days later during development, TAG-1 immunoreactivity is present in fibers of the IZ, CP and MZ of the basolateral cortex: in the IZ, the TAG-1 immunopositive fibers are tangentially oriented, projecting toward the internal capsule, while distinct fascicles of axons cross the whole thickness of the CP (figure). At later stages (E17.5), TAG-1 immunoreactivity is still detectable in the IZ, CP, and MZ but in the dorsomedial rather than basolateral cortex of Emx1 knockout and wild type embryos (not shown).

L1 is another cell adhesion molecule involved in axon outgrowth and pathfinding; it is also a well-established marker of thalamocortical axons since the beginning of their fasciculation.

We use L1 antibody to specifically stain thalamic axons, and found that these projections were not affected in $Emx1^{-/-}$ brains (figure 3.22, B).

3.7 MORPHOLOGICAL AND NEUROCHEMICAL ANALYSIS OF THE ADULT $Emx1^{-/-}$ CEREBRAL CORTEX

We have examined brains and in particular cortices of adult Emx1 null mice using standard histological methods as well as immunocytochemical techniques with cell-specific markers.

First of all, we generated mice with the three Emx1 genotypes (homozygous mutant, heterozygous and wild type), intercrossing heterozygous animals and allowing the pregnant females to give births.

The $Emx1^{-/-}$ mice were viable and grew to adulthood; they were also fertile. Moreover, these mice did not exhibit any obvious behavioural alteration.

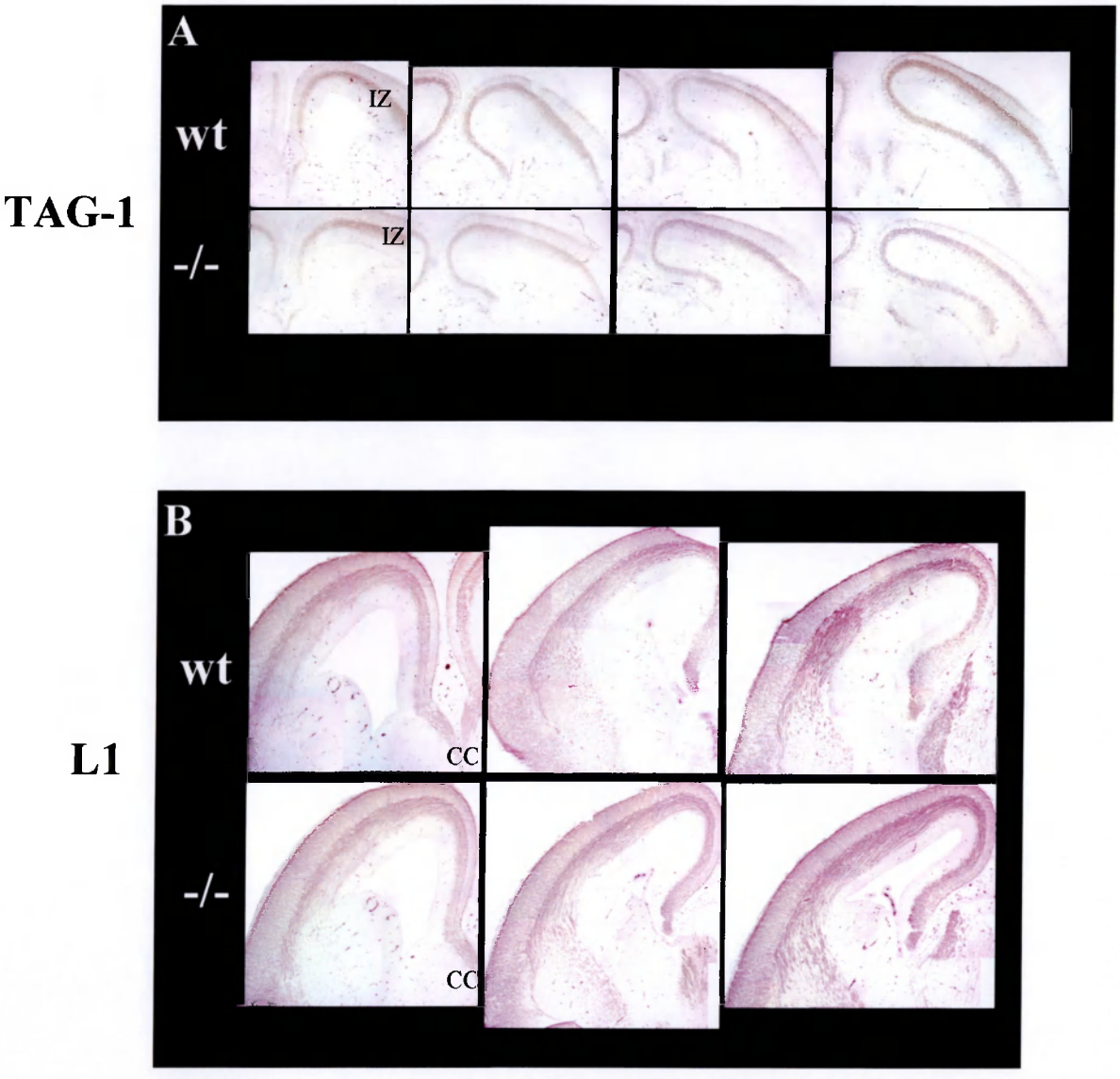


Figure 3.22

Tag-1 (A) and L1 (B) immunocytochemistry on frontal sections of E15.5 normal (wt) and Emx1$^{-/-}$ embryos. (A) Labelled axons are oriented tangentially to the IZ and directed toward the ventral telencephalon. (B) Labelled axons are directed toward the dorsal and the middle telencephalon, tangentially to the cortical surface. CC, corpus callosum; IZ, intermediate zone.

3.7.1 The anatomy of $Emx1^{-/-}$ cortices

We studied the general anatomy of 20 $Emx1^{-/-}$ adult (8-12 weeks old) brains and the same number of $Emx1^{+/-}$ and $Emx1^{+/+}$ littermates.

First, we could not detect any difference in size and weight by comparing adult $Emx1^{-/-}$ mice with $Emx1^{+/+}$ littermates. As an indication, in figure 3.23 it is reported a diagram showing a comparison between the mean values (±S.M.E.) of the brain weights of ten Emx1 null mice and the same number of wild type littermates. We could not also detect significant differences by comparing the sizes of those brains (not shown).

Subsequently, we performed an extensive analysis of $Emx1^{-/-}$ brains, by observing under the light microscope histologically stained sagittal, frontal and coronal brain sections.

We found that all brain structures were normal, confirming that not only the embryonic development of the CNS occurs without disturbances, but also alterations or degeneration do not even occur later during the post-natal development and the adult life.

To date, the presence of a normal corpus callosum was always detectable in the mutants as well as in the wild type controls (arrows in figure 3.24); morphological defects were not found in the hippocampal formation (figure 3.24) and in the olfactory bulbs.

Moreover, we found that the overall cytological appearance of the cerebral cortex, including the organization of the six cortical layers (figure 3.24) was indistinguishable between the mutant mice and the wild type littermates. We measured the thickness of the cerebral wall in order to verify if there was an even subtle defect in the cellular density of the cortex, resulting at a first glance as an alteration in the cortical thickness, but we could not detect any difference (data not shown).

3.7.2 The cytology of $Emx1^{-/-}$ cortices

Emx1 gene is expressed in adult cerebral cortex. In particular, it has recently been shown that this gene is exclusively expressed by pyramidal neurons from the early stages of embryonic life to adulthood (Chan et al., 2001).

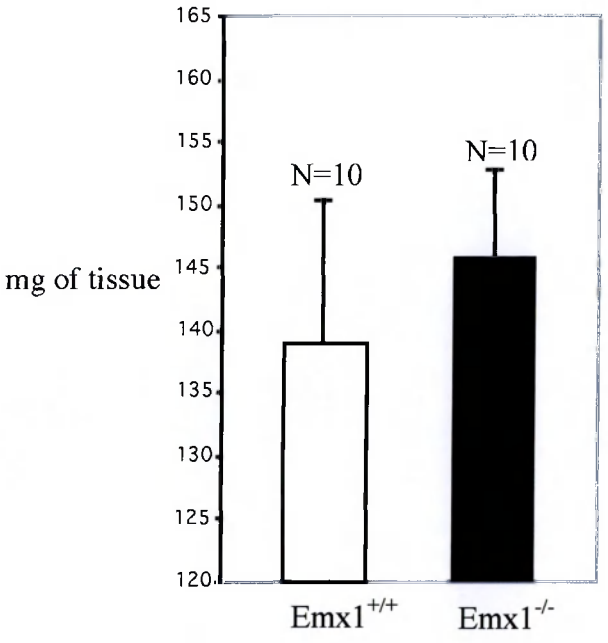


Figure 3.23
Diagram showing a comparison between the weight of wild type adult brains (white bar) and knockout one (black bar). Bars indicate the mean value calculated on a total number of 10 samples for each genotype ± S.E.M.

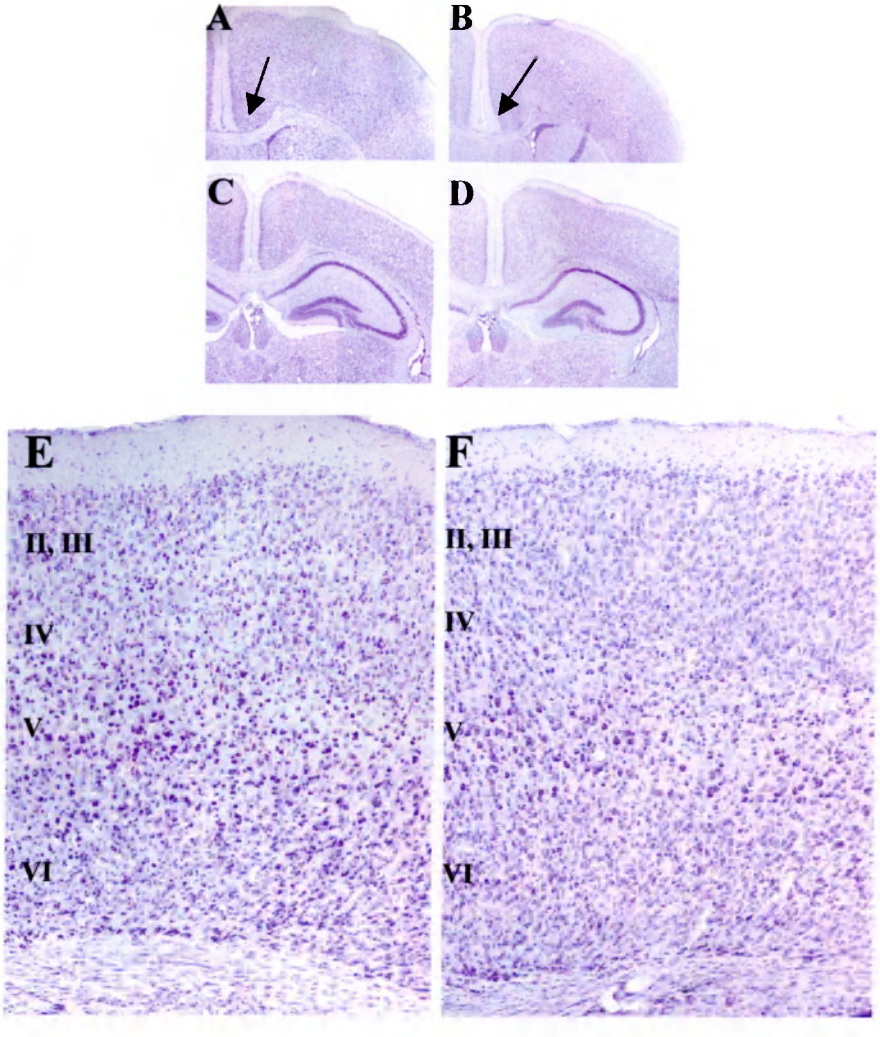


Figure 3.24
Nissl-stained frontal sections of 12 weeks old Emx1-/- (B, D, F) and wild type (A, C, E) brains. In high magnification pictures of the cerebral cortex, it is possible to distinguish by cell morphology and density the six layers (E, F).

The finding that Emx1 is expressed in pyramidal neurons both in developing and mature cortex led us to hypothesise that this gene could be involved in the specification and maintenance of the pyramidal phenotype.

As reported above, pyramidal neurons are glutamatergic projection neurons that constitute the majority of cortical cells and originate in the cortical ventricular zone; the remaining neurons of the cortex are the non-pyramidal cortical interneurons that use GABA as neurotransmitter and originate in the ventral telencephalon.

We performed a series of experiments aimed to analyse the density and the laminar distribution of these two neuronal cell types in the adult cortex of the mutant mice compared with wild type littermates.

We used immunocytochemistry with antibodies against glutamate and GABA, markers of the two neuronal cell types.

In agreement with earlier reports, we found that glutamate-labelled neurons were present in all cortical layers except layer I, whereas GABA-containing cells were scattered throughout the cortical thickness in both mutants and wild type mice (figure 3.25).

Subsequently, we counted the total number of cells labelled with anti-glutamate and anti-GABA antibody respectively, in frontal sections taken at different levels along the rostro-caudal axis (rostral, medial and caudal corresponding to motor, somatosensory and visual cortical areas, respectively)

For anti-glutamate immunostained sections, we standardised a rectangular area, of 200μm base and variable height according to the depth of the cortical wall (as already reported, the thickness of the cerebral wall was never significantly different between the mutant and the wild type brains), which was placed across the cerebral cortex and perpendicular to the fibers of the corpus callosum. Whereas for GABA-stained cells, we used a rectangular area of 1mm base. For each brain sample we counted cells in three comparable sections at each rostrocaudal level, and calculated the mean of the three values.

Cells counts revealed no significant difference in the number of pyramidal neurons between the two groups of animals; in figure is shown the result of the counts performed on 8-weeks old both mutant and wild type brains (figure 3.26 A).

However, counts of GABAergic neurons showed statistically significant ($p<0.05$, two tailed Student's t test) differences between mutant and wild type

littermates, but only at the level of somatosensory and visual cortical levels. Specifically, these cortical areas in $Emx1^{-/-}$ mice showed a significantly reduced number of GABA-containing neurons; the density of these cells was similar at rostral levels in the two groups of animals (figure 3.26 B).

3.7.3 Origin of the GABAergic cell defect in Emx1 deficient mice

To elucidate the mechanisms that lead to a diminution in the number of GABA-containing cortical interneurons in $Emx1^{-/-}$ mice, we investigated their origin during development.

As already reported, cortical GABAergic interneurons originate mostly in the medial ganglionic eminence (MGE) of the ventral telecephalon and follow tangential migratory routes to reach the dorsal telencephalon, by interacting with TAG-1 expressing axons of the developing corticofugal system.

In order to investigate if Emx1 gene inactivation influences this process, we studied the expression pattern of molecular markers specific of tangentially migrating cells, such as Calbindin and Lhx6, during embryogenesis.

Lhx6 is a Lim-homeobox containing gene expressed in the subventricular and submantle zones of the mouse MGE from E11.5 and E17.5 (Lavdas et al., 1999), but it is also expressed in a specific subset of cells of the developing neocortex. In particular by E15.5 the number of Lhx6-expressing cells in the MGE is greatly reduced, but expression of this gene is seen in cells of the MZ, SVZ and IZ of the developing cortex. It has been shown that those cortical Lhx6-expressing cells are GABAergic interneurons tangentially migrating from MGE into the neocortex (Lavdas et al., 1999).

We used immunocytochemistry with antibodies for Calbindin and Lx6 (a generous gift from V. Pachnis) on frontal sections of E15.5 and E17.5 Emx1 mutant and wild type brains. We found that at these ages, as previously reported, both calbindin and Lhx6-positive cells were located into the neocortex mainly in the MZ, IZ and subplate; a small number of cells was also scattered into the forming CP. No differences were found between Emx1 mutant and wild type littermates in the 80% of cases (figure 3.27 and 3.28).

However, 20% of samples showed a misrouting of tangentially migrating cells, consisting in the lack of the three typical bands of cells, that instead spread randomly

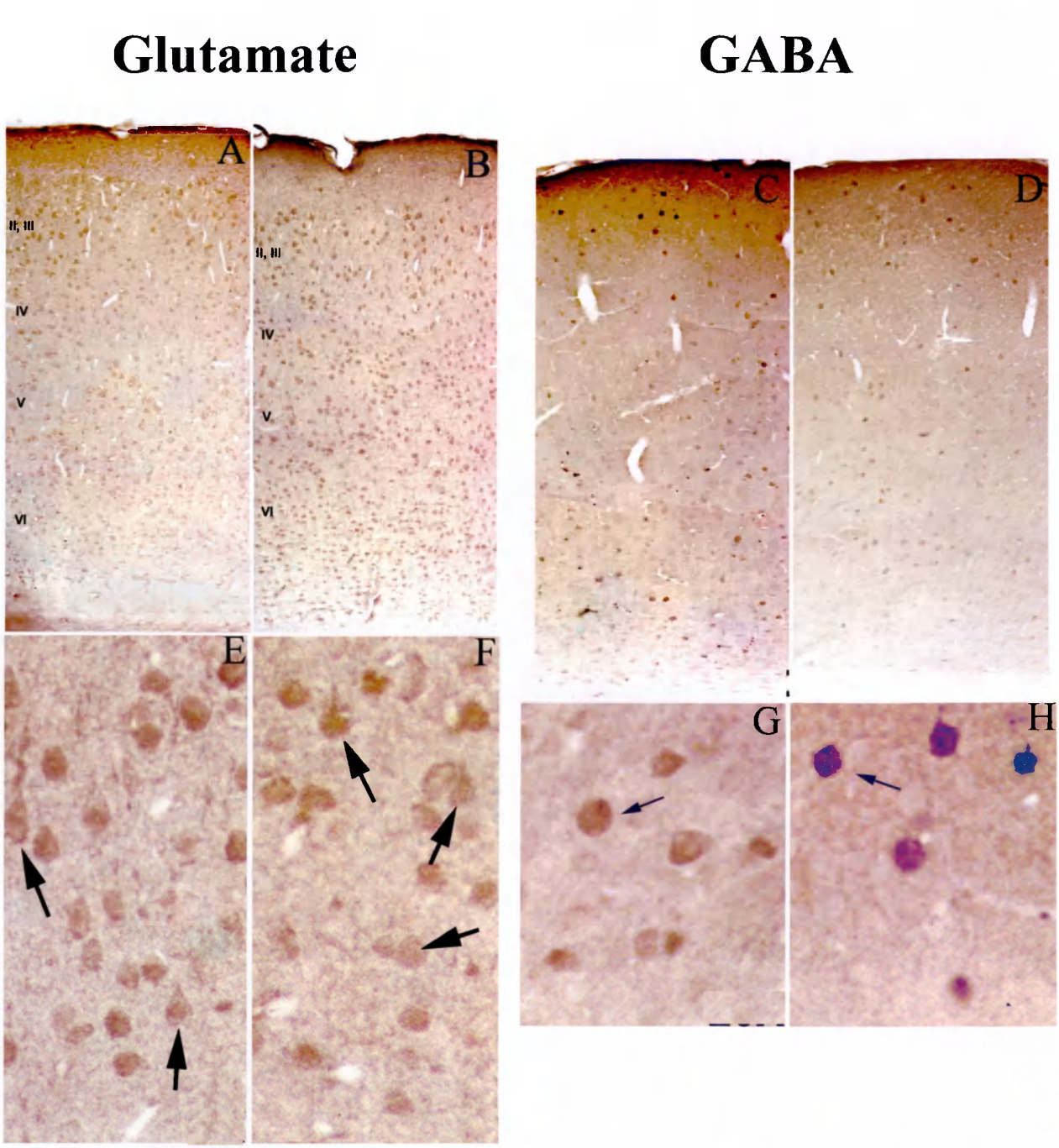


Figure 3.24

GABA and glutamate staining in the cerebral cortex of Emx1$^{-/-}$ and wild type adult mice. The morphology (arrows in E anf F point to cortical pyramidal neurons that have also the apical process in the plane of the section) and the distribution of glutamate-containg cortical cells are similar between wild type and Emx1 mutant cortices. GABAergic cells are scattered troughout all cortical layers and show a rounded morphology (arrows in G and H).

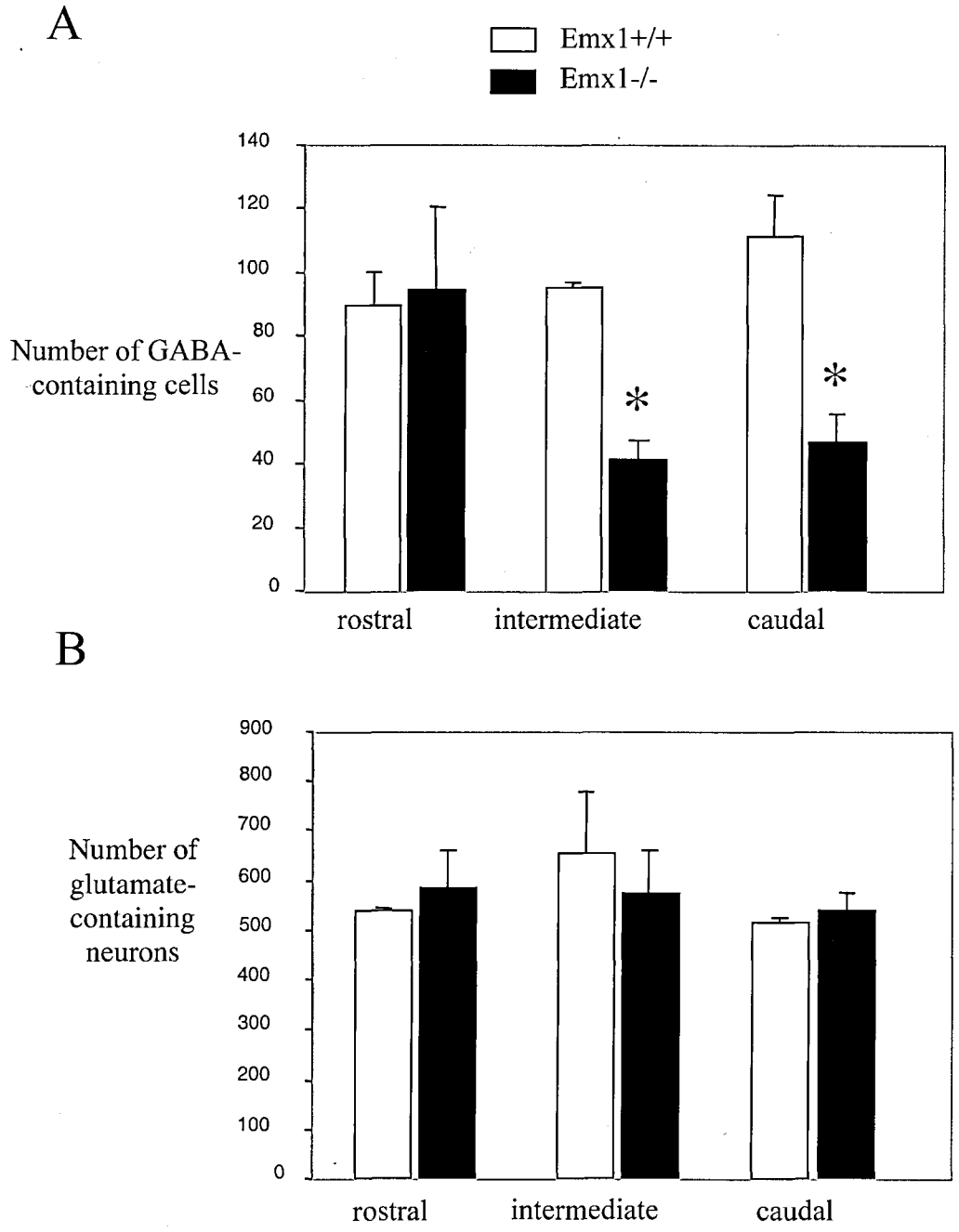


Figure 3.26

Diagrams showing counts of GABA- (A) and glutamate-containing (B) cells at 3 levels of the cortex along the rostro-caudal axis (rostral, intermediate, caudal) in $Emx1^{-/-}$ and wild type adult brains. (A) The graph shows a significantly reduced number of GABAergic neurons in $Emx1^{-/-}$ (black boxes) mice at intermediate and caudal areas, but not in the rostral cortex. (B) Cell counts at 3 levels of the cortex along the rostro-caudal axis show no significant differences in the number of glutamate neurons in the two groups of animals . For each labelling and each genotype four knockout mice (8 weeks old) and four wild type littermates were analysed.

into the cortical wall and/or are clearly reduced in number (figure 3.28).

This percentage was too low to justify the diminution of GABAergic interneurons observed in adult Emx1 mutant cortices. In fact, cell counts of GABA-containing cortical interneurons at P0 did not reveal any significant difference between $Emx1^{-/-}$ and wild type littermates (not shown).

Therefore, we hypothesised that the significant loss of these neurons occurs later after birth. On the other hand, the abnormalities observed during development might represent a very low penetrant consequence of the Emx1 gene deletion, and might only partially contribute to the loss of GABAergic interneurons that we observed in the adults.

As a confirm, we could not detect defects in any other component of the tangential migratory machinery, including the TAG-1-expressing axons of the corticothalamic system, shown to be essential for the proper influx of cells into the developing neocortex.

Finally, we have recently started to investigate if the loss GABAergic interneurons in adult Emx1 mutant cortices corresponds to an increase in the apoptotic cell death, using TUNEL.

Preliminary results indicate that in $Emx1^{-/-}$ cortices the density of apoptotic cell bodies is comparable to that of the wild type ones.

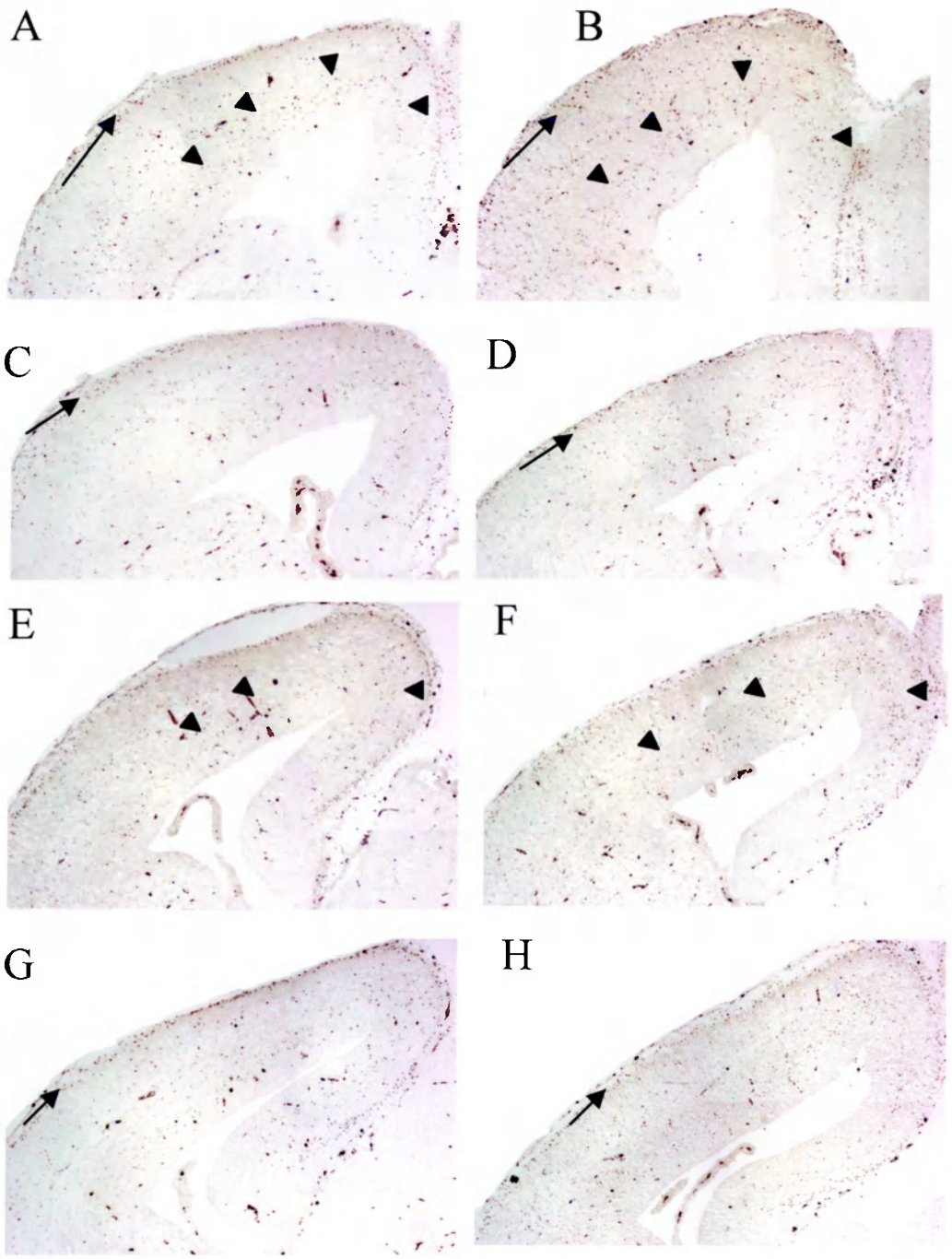
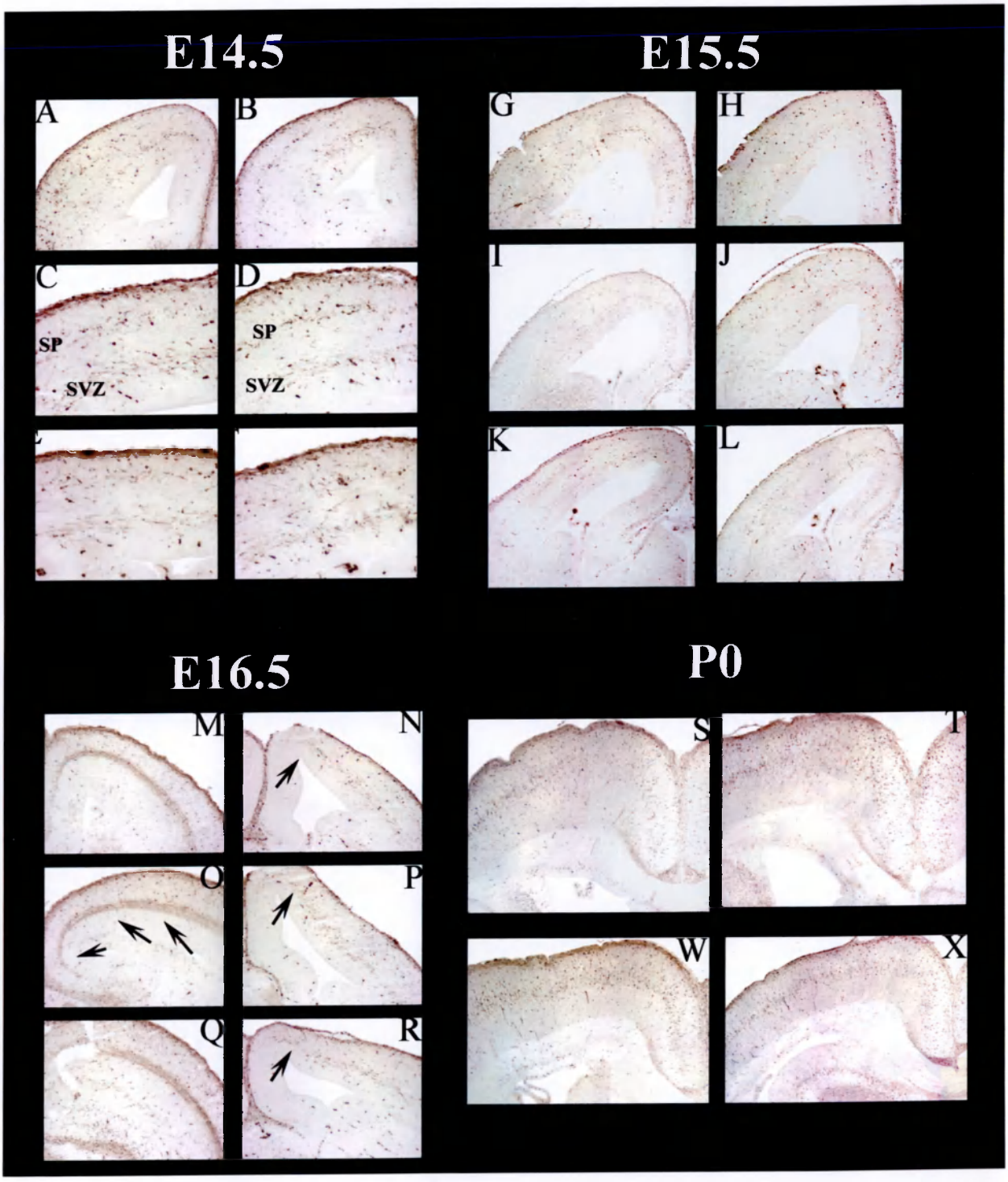


Figure 3.27

Lhx6 immunocytochemistry on E15.5 frontal sections of a wild type (B, C, E, G) and mutant (B, D, F, H) brains from rostral to caudal. Arrows indicate the Lhx6-positive cells in the MZ, whereas arrowheads point to the cells in the intermediate zone.


E14.5
E15.5
E16.5
P0
A
B
C
D
SP
SVZ
SP
SVZ
G
H
I
J
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L
M
N
O
P
Q
R
S
T
W
X

Figure 3.28

Calbindin immunocytochemistry on frontal sections of brains, both mutant and wild type taken at different embryonic ages (A-R) and at P0 (S-X). A-F, E14.5 frontal sections of a wild type (A, C, E) and a mutant (B, D, F) brain: three bands of calbindin-positive cells are visible, corresponding to the MZ, SP and SVZ, both in the wild type (B, E) and in the mutant (C, F) brain. At E15.5, the distribution of calbindin-positive cells in the mutant brain (H, G, L) is comparable to that of the wild type brain (G, I, K). N, P, R are sections of a E16.5 mutant brain showing an abnormal pattern of calbindin-containing cells: the density of these cells is lower, particularly in the medial portion of the cortex (arrows in N, P, R), compared to that of the control (M, O, Q). Moreover, at this embryonic stage, calbindin is also detectable along the thalamic axons projecting into the wild type neocortex (arrows in O). However, it is not possible to detect any anti-calbindin staining at this level. At P0 (S-X) no difference is seen in the distribution and the density of calbindin-positive cells comparing Emx1 deficient brains (T, X) with the wild type ones (S, W).

3.8 NEURONAL HYPEREXCITABILITY IN Emx1$^{-/-}$ MICE

Earlier studies have provided evidence in support to the hypothesis that loss of GABAergic neurons results in neuronal hyperexcitabilility in the cortex, that eventually leads to seizure activity (Ribak et al., 1979, 1982).

Moreover, it has been shown by using animal models that disruption of GABAergic neurotransmission is implicated in epilepsy.

These reports have led us to hypothesis that the observed significant loss of GABAergic neurons in the cortex of Emx1$^{-/-}$ mice would result in neuronal hyperexcitability leading to seizure activity.

We obtained baseline EEG recordings from 8 free-moving knockout mice and the same number of wild type littermates (figure 3.29, G, H).

Examination of these recordings showed epileptic-like activity characterised by abnormal spikes in all Emx1$^{-/-}$ animals that have been analysed.

The immediate early gene *c-fos* is a well-established marker of neuronal activation in the brain. To identify the brains regions and the cell types affected by epileptic seizures in the mutant mice, we decided to stain with the antibody against *c-fos,* brain slices derived from the animals previously recorded and subsequently killed (figure 3.29, A-F).

We found increased labelling of cells in the cortex and in various sub-fields of the hippocampal formation. The density and disposition of the staining suggested that both projection neurons and interneurons are affected (figure 3.29).

These results confirmed our hypothesis about a possible alteration of the neuronal circuits and therefore electrical activity in Emx1$^{-/-}$ brains.

3.9 EPILEPTIC PATIENTS AND EMX1 GENE SEQUENCING

To gain insights into the possible involvement of Emx1 gene in human epilepsy, we decided to analyse DNA from epileptic patients, using PCR and subsequently sequencing the entire coding region of the gene.

We had in our laboratory a collection of DNA samples from patients with different neurological diseases; among these, there were samples from patients affected by sporadic or inherited epileptic syndromes.

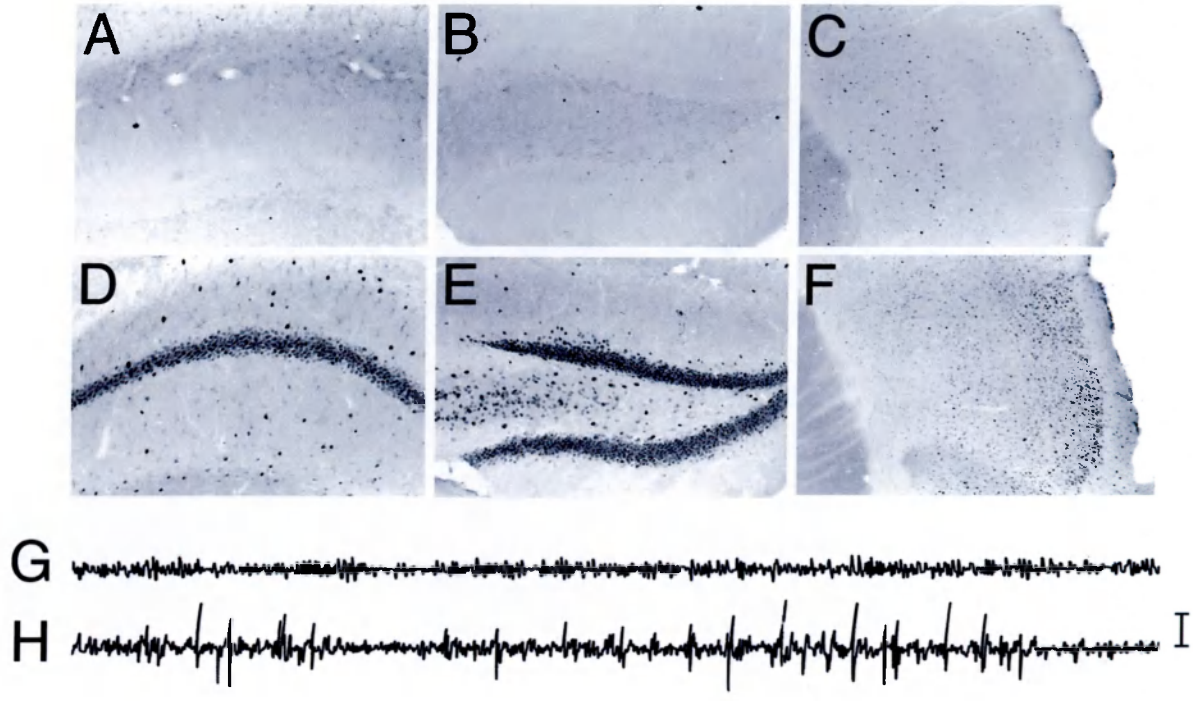

Figure 3.29

c-fos immunoreactivity and EEG recordings from Emx1$^{-/-}$ and wild type adult mice. *a-f, c-fos* immunoreactivity in hippocampal subfields (CA1, *a,d*; dentate gyrus, *b,e*) and in the perirhinal cortex (*c,f*) of wild type (*a-c*) and knock-out mice (*d-f*). Note that immunoreactivity is significantly increased in pyramidal and granule cells of the hippocampus and in the external layers of the perirhinal cortex in *Emx1*$^{-/-}$ mice. *g,h*, representative EEG tracings from the hippocampus of wild type (*g*) and knock out (*h*) mice. Calibration bar: 20 μv.

We also collected other blood samples from patients affected by different types of epileptic syndromes as well as from unaffected individuals (controls), residing in different regions of Italy from Southern to Northern.

We have previously designed three couples of oligonucleotide primers that allow the amplification of both exons and intron-exons boundaries in the Emx1 gene. So we were able to perform PCR amplification of the entire coding region of the gene, purify PCR products (not shown) from agarose gel and analysed them on an automatic sequencer (see Materials and Methods).

We found a point mutation in the second exon of EMX1 gene in seven epileptic members of a family affected by idiopathic generalised epilepsy and in another affected member of a second family, characterised by symptoms of the same epileptic syndrome.

3.9.1 The affected families

The first family, whose epileptic members were diagnosed as having the mutation in EMX1 gene, was from the North-eastern Italy and had a five-generation pedigree. The second family was composed only by four member (two affected and two unaffected), was from North-western Italy (figure 3.30)

The first family showed two distinct epileptic phenotypes. Both phenotypes may be included in the group of idiopathic generalized epilepsies (IGEs) on the basis of the normal intellect, the clinical and neuroimaging findings and the EEG pattern.

Three subjects (III-6, III-9 and III-10) were diagnosed as having juvenile myoclonic epilepsy (JME) because of an adolescent (III-9 and III-10) and early-adult onset (III-6) of myoclonic jerks at awakening and generalized tonic-clonic seizures (GTCS), normal EEG background with typical generalized paroxysms, clinical photosensitivity and good response to valproate.

Six members (II-3, III-1, III-7, III-8, III-38 and IV-21) of the family showed clinical and EEG features indicative of an epilepsy with pure GTCS (EGTCS).

Other three members (IV-8, IV-23 and IV-24) reported only febrile seizures during infancy.

Two members of the smaller family (figure 3.30, A) manifested rare pure GTCS in adult age. The clinical information and EEG picture was indicative of EGTCS.

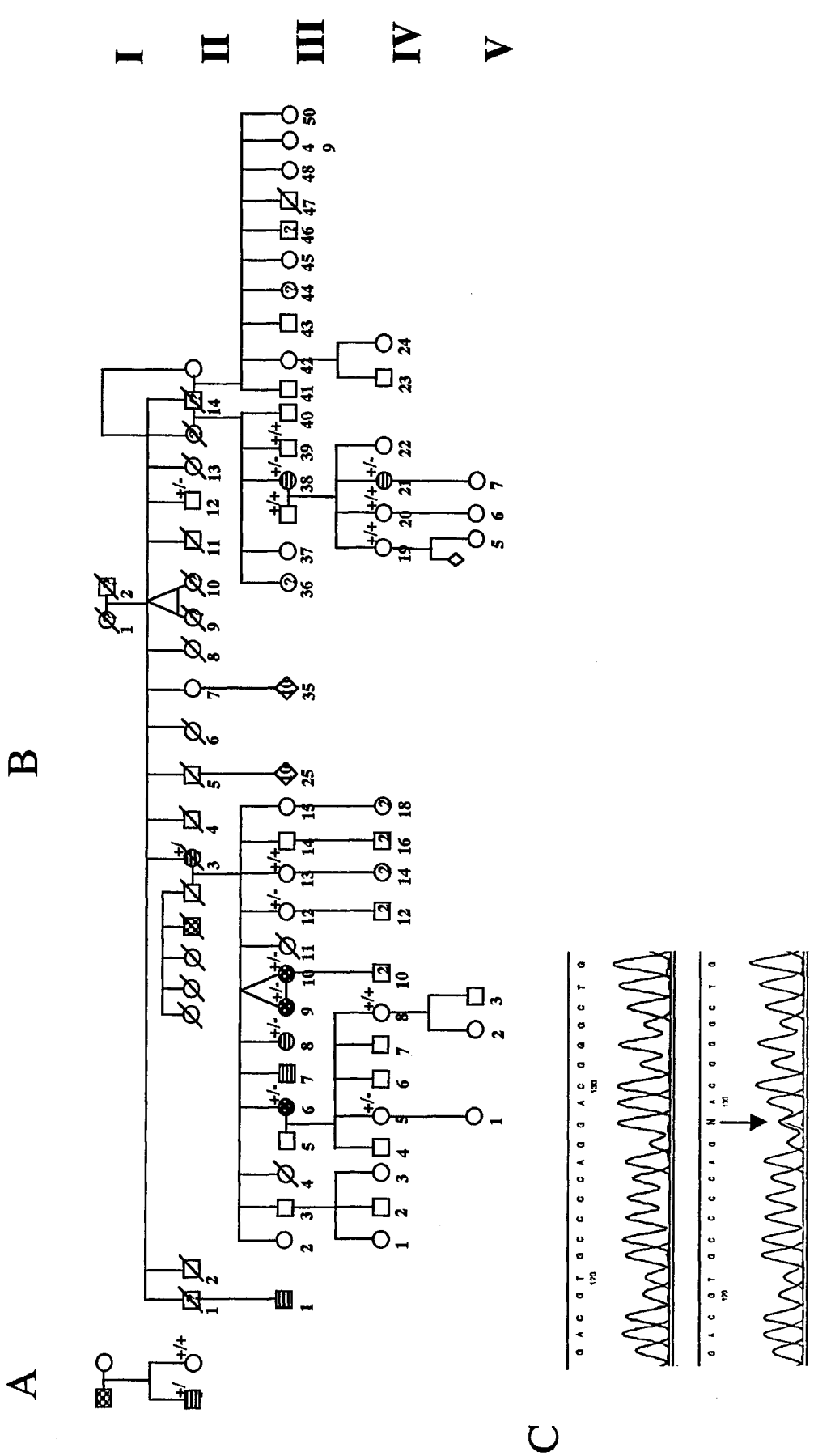
A
B
C
I
II
III
IV
V

Figure 3.30

A, B, Pedigrees of two and five generation families with IGE. The '?' symbol indicates no clinical and genetic information. The individuals showing the symbols '+/+' or '+/-' were genotyped. The'+/+' symbol indicates homozygous wild type genotyped individuals; whereas the '+/-' symbol indicate heterozygous individuals.


Unaffected individuals


Unclassified epilepsy


Febrile seizure


Juvenile myoclonic epilepsy


Epilepsy with generalized tonic-clonic seizures only

C, Sequencing trace of the second exon of *EMX1* gene. The position of the base change is shown on the lower part (arrow); "N" in the nucleotide sequence indicates the G→A substitution. The wildtype sequence is also depicted (upper).

3.9.2 The EMX1 gene mutation

The affected members of the two families had a mutation at +18 bp of the second exon of EMX1. This mutation results in a heterozygous substitution G→A that generates a change D→N in the amino acid residue in affected patients II-3, III-6, III-8, III-9, III-10, III-38 and IV-21 (figure 3.30). Unaffected individuals II-12, III-12 and IV-5 also showed the same mutation (figure 3.30). However, unaffected individuals III-13, III-39, IV-8, IV-19, IV-20 did not exhibit the gene mutation.

The analysis of DNA samples from the smaller family (figure 3.30 A) revealed that one of the two affected members showed the previously described substitution G→A. The DNA from the other affected individual was not available.

No mutations were found in 11 DNA samples from sporadic cases of IGE.

420 control chromosomes obtained from normal Italian individuals were sequenced and no mutations were detected (figure 3.30, C).

All members of the two families with a defined IGE syndrome (JME or EGTCS) were heterozygous for the EMX1 mutation (except for III-1 and III-7 of the large family for whom genomic DNA was not available).

The EMX1 mutation and the two IGE phenotypes co-segregate and show a pattern consistent with autosomal dominant inheritance. The lack of epileptic phenotype in three heterozygous members (II-12, III-12 and IV-5) could be explained by incomplete penetrance of the EMX1 mutant phenotype or, in the case of IV-5, by the relatively young age.

The sequence analysis of the first and third exons of the gene did not revealed any mutation in the affected and unaffected individuals of the two families as well as in the controls.

The PHD protein structure prediction for the EMX1 protein secondary structure (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) did not reveal strong changes in the folding caused by the mutation, except for a lower probability to get a α-helix in the region where the substitution occurs.

Finally, the Emx1 sequence analysis among different species revealed that the base A, at the level of which the substitution occurs, is strongly conserved during evolution.

QUANTIFICATION OF THE RELEASE OF AMINO ACID NEUROTRANSMITTERS IN Emx1 KNOCKOUT BRAINS

We have found that Emx1 null mice exhibit an epileptic-like phenotype presumably due to a reduction of cortical inhibitory interneurons. On the other hand, Chan et al. (2001) have recently showed that Emx1 gene is specifically expressed by pyramidal glutamatergic neurons in the rodent cerebral cortex.

We, therefore, wanted to investigate whether a reduction in the number and density of GABAergic neurons would have changed neuronal circuits and the neurotransmission properties in $Emx1^{-/-}$ cerebral cortex.

We started a series of experiments for simultaneously measuring the quantity of all amino acid neurotransmitters that are released by cortex crude synaptosomes in superfusion (for further details see Materials and Methods). We chose to look at the occipital cortex because our previous results indicated a significant reduction of GABAergic cells only in the medial-caudal part of the mutant cerebral cortex.

We prepared crude synaptosomes from occipital cortex of 6 Emx1 null adult (10 weeks old) mice and the same number of wild type littermates; we measured both the spontaneous efflux of GABA and the quantity released after stimulation with 50mM KCl.

We have found a significant decrease (25%) of both spontaneous and depolarization-evoked efflux of GABA in mutant mice in comparison with the wild-type ones (figure 3.31). Interestingly, we have also observed a marked decrease (40%) in the basal and evoked efflux of aspartate and glutamate, the two major excitatory amino acid neurotransmitters, often reported to be co-localized and co-released by neuronal cells (figure 3.31).

All these data indicate that in Emx1 knockout mice some mechanisms of neurotransmission are affected, and might explain the altered, epileptic-like electric activity recorded in the cortex of these animals, given the key role that the inhibitory amino acid plays in balancing neuronal excitation throughout the CNS.

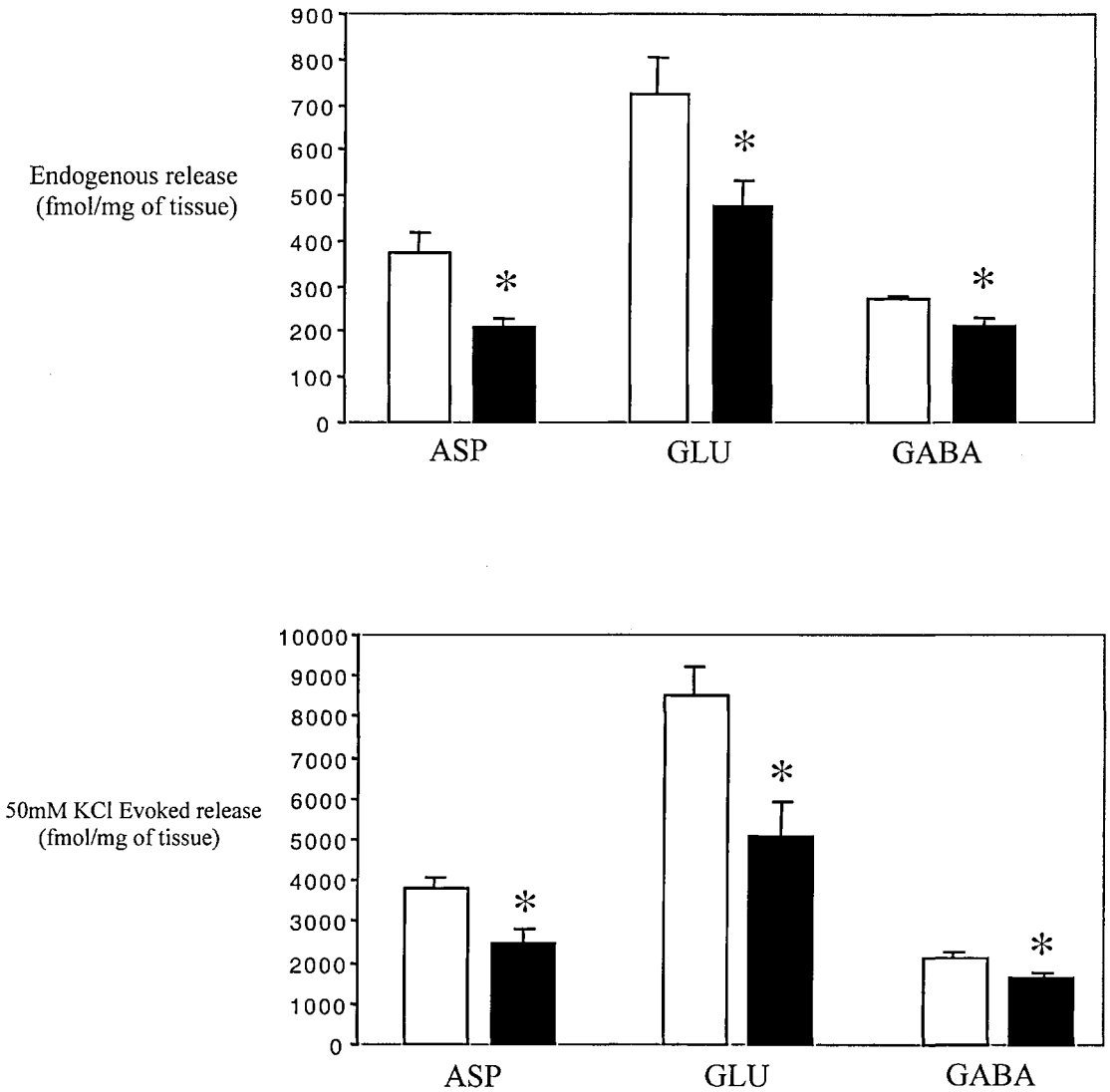

Figure 3.31

Release of endogenous amino acids from wild type and Emx1 knockout mice. The figure shows the spontaneous (upper panel) and evoked (KCl 50 mM; lower panel) release of aspartate, glutamate and GABA from mice occipital cortex synaptosomes in superfusion. Each bar represents mean ± S.E.M. of 13-27 observations from 6 wild type (white bars) and 6 Emx1 null mutant (black bars) mice. Both basal and evoked release of the three neurotransmitters was significantly lower in the knockout than in control mice. *$p<0.05$ at least, two-tailed Student's t test.

Chapter 4

DISCUSSION

Emx genes represent powerful tools for studying the developing brain and, in particular, the developing cerebral cortex. Their expression patterns during mammalian embryogenesis allow us to closely follow major events in cortical neurogenesis and differentiation.

It is also conceivable that mutational events in these genes or some of their targets underlie a number of brain defects. These considerations led us to investigate the functions of these genes, and in particular of Emx1, during cortical development and even later when that cortex is mature.

The results obtained from such an analysis not only gain insights into the molecular mechanisms underlying brain and cortical development, but also appear of primary relevance toward the understanding the nature of brain defects such as epilepsy.

4.1 THE DEVELOPMENT OF EMX1 NULL MUTANT MICE

We investigated the development of the central nervous system and, in deeper details of the cerebral cortex, in mice lacking a functional Emx1 gene.

Emx1 homeobox gene is expressed in the developing and mature cerebral cortex. Particularly, during development, Emx1 is expressed not only in cells of the ventricular zone, like Emx2, but also in postmitotic neurons that will later form the six layers of the adult cerebral cortex.

Thus, Emx1 might play critical roles in proliferation, migration and differentiation of the cerebral cortex.

To date, both Emx genes have been implicated in mouse forebrain development; in fact, the anatomical disruptions following Emx2 deletion are profound and have been well studied. By contrast, very little is known about the role of Emx1.

Emx2 knockout mice show significant defects, including reduced size of the cerebral cortex and hippocampus, absence of the dentate gyrus, impairment of radial migration and alterations in the specification of cortical area identities. On the other hand, according to previous reports, the brain structural alterations of Emx1 null mutant mice are subtle.

However, the expression pattern of Emx1 gene suggests potential roles not only in brain developmental mechanisms, but also in the specification and maintenance of some cortical properties.

4.1.1 Emx1 *loss of function* mice have a normal corpus callosum

It has been reported that Emx1 mutant mice, made either with 129-strain derived ES cells (Qiu et al., 1996) or ES cells derived from an F1 embryo between C57BL/6 and CBA mice (Yoshida et al., 1997), lack most or all of their corpus callosum.

However, in the last few years it has come to light that the genetic background can exert important roles in the genesis of the corpus callosum in mutant mice generated by ES cells technology.

Therefore, we backrossed our Emx1 mutant mice (derived from those of Yoshida et al., 1997) into the C57BL/6 background, and found that Emx1 homozygous mutant mice with C57BL/6 genome have a normal corpus callosum.

Our results suggest that the mutation of the Emx1 gene does not contribute directly to the acallosal or discallosal phenotype associated with Emx1 mutant mice derived from a 129/Sv or a mixed C57BL/6-CBA background.

Moreover, Gou et al. (2000) have published results of a similar analysis, that are in accord to our unpublished observations.

It has also to be mentioned that according to Yoshida et al. (1997), Emx1 null mutant mice exhibit other subtle brain abnormalities: cortical layers are poorly differentiated and half of these mutant mice die neonatally for unknown reasons.

By contrast, we found that $Emx1^{-/-}$ mice with C57BL/6 background are born in a Mendelian ratio and grow to adults; moreover they exhibit well-differentiated cortical layers.

It might therefore be that the mixed genetic background, which contains a percentage of CBA genome, underlies the observed phenotypic differences.

Finally, our results indicate that Emx1 gene is not directly involved in the formation of indusium griseum and tenia tecta.

4.1.2 Anatomical and molecular changes associated with the development of the neocortex are not altered by Emx1 gene deletion

We followed the formation of the neocortex in the absence of a functional Emx1 gene utilizing histological and immunocytochemical methods as well as BrdU pulse-labelling experiments.

By E10.5, the mutant forebrain is undistinguishable from the wild type one, not only morphologically but also neurochemically, meaning that the processes of neocorticogenesis is not affected by the Emx1 inactivation.

We used specific molecular markers to visualize proliferating neuroblasts as well as migrating and differentiating cortical neurons. Moreover, we pulse-labelled timed pregnant female in order to investigate the rate of cell proliferation and the dynamics of neuronal radial distribution into the layers of the forming cortical plate.

At even later stages of cortical development, all parameters utilized to follow the neurogenesis in the mutants were identical to those of the wild type littermates: in other words, processes of neuroblasts proliferation, migration and settling of post-mitotic neurons in the forming cortical plate normally occur in the mutants.

As a result the cortical architecture in the Emx1 null mutant both early postnatal and adult mice is normal.

Nevertheless, during cortical neurogensis the only difference that we were able to detect in $Emx1^{-/-}$ mice was at the level of the subplate. However, this defect does not correlate with any other abnormality of the developing cortex.

4.1.3 The subplate is poorly differentiated in the cortex of Emx1$^{-/-}$ mice

Morphological and neurochemical examination of sections from cortex of Emx1$^{-/-}$ embryos and newborn mice have shown that the subplate is reduced in thickness or absent from the medial cortex and, more prominently from medial-caudal portion of the cortex.

In particular, this was evident in Nissl-stained sections and in sections stained for calretinin, a marker of subplate neurons during embryogenesis.

However, analyses performed with other molecular markers such as Tbr1, Map2, and Calbindin did shown any strong difference in the mutant subplate compared to the wild type one. We also could not detect abnormalities in the thalamic axons that innervate the neocortex upon interactions with subplate cells. This indicates functions of the subplate neurons are unaltered.

It might, therefore, be that only a subpopulation of subplate cells is affected by the Emx1 gene deletion, and more specifically the calretinin-containing cells of a specific rotrocaudal and lateromedial area of the subplate.

The specific role played by these subset of neurons is not known; however, it might be related to the guidance of incoming cells from the ventral telencephalon into the neocortex. Indeed, we observed that in more severe cases these defects are related to a misrouting of tangentially migrating GABAergic interneurons.

Finally, the absence of subplate cells from the medial and caudal portion of the cerebral cortex correlates with the graded expression of Emx1. During development, in fact, this gene is more prominently expressed in this part of the cortex, where the subplate defect is more pronounced in the mutants.

Interestingly, this finding suggests a possible analogy with Emx2: knockout mice lacking a functional Emx2 gene exhibit subplate defects that are restricted to caudal and medial subplate, where normally the gene has a maximum of expression.

One explanation could be related to the fact that subplate neurons, even if sharing some markers, are not homogeneous. Therefore, it is likely that at different location along both main tangential axes, different doses of both EMX1 and EMX2 might be necessary to their VZ neuroblasts for initiating their specific and different morphogenetic programs.

If some functional interchangeability takes place between EMX2 and EMX1, it is conceivable that in more rostral and lateral neuroblasts, the absence of EMX1 can be

rescued by EMX1 protein and viceversa. On the other hand, this would not be possible in more caudal and medial neuroblasts because of the very high, unrescuable levels of EMX proteins that these cells require.

4.1.4 Emx1 does not directly contribute to the specification of cortical area identities

The analysis of area-specific expressed genes, such as Cad6, Id2, Id3, and Lamp, has not revealed any alteration in the absence of a functional Emx1 gene, suggesting that the gene is involved neither in the specification nor in maintaining neocortical area identities.

On the other hand, our *in situ* hybridization experiments show that COUP-TF1, a factor recently shown to have an essential role in the specification of cortical areas, is overexpressed in Emx1-/- cortices, even if its spatial domain of expression is not altered.

In particular, COUP-TF1 is responsible for the regional expression of many genes as well as for the appropriate projection of thalamocortical axons into the neocortex.

These findings rise several question. First, is Emx1 an upstream negative regulator of COUP-TF1? Moreover, what is the effect of this increased expression of COUP-TF1? Does it work to compensate the absence of Emx1 and therefore to ensure a normal specification and maintenance of cortical areas?

This scenario is even more complicate, considering that COUP-TF1 is also supposed to have a role in the development and maintenance of subplate neurons; in fact, in the absence of a functional COUP-TF1 gene, subplate defects evolve resulting in abnormal thalamocortical projections.

One possible explanation could be that COUP-TF1 is a redundant or compensating factor, which in the absence of a functional Emx1 gene, ensures a normal functionality of subplate and provides infomation for the proper thalamocortical innervation of the cortex, that we observe in mutants.

However, these are still open questions. We need to set up new experiments to try to elucidate the interplay between Emx1 and COUP-TF1.

4.1.5 Normal cortical and thalamic projections in the cortex of $Emx1^{-/-}$ mice

The expression of Emx1 gene across the entire thickness of the developing cerebral cortex as well as the defects observed in the subplate in $Emx1^{-/-}$ animals, suggest

that Emx1 gene deletion might alter the patterns of corticofugal and thalamocortical connections.

Thalamic axons send their projections to innervate the cortical plate and provide afferent inputs. Development of proper thalamocortical projections involves two processes: the guidance of axon projections to the appropriate cortical regions, followed by innervation of the cortical plate. Ablation of subplate neurons leads to the failure of thalamocortical axons to stop at their appropriate targets into the neocortex (Ghosh et Shatz, 1993). Subplate cells in this process are supposed to provide guidance cues for thalamocortical axons (Molnar and Blakemore, 1995).

On the other hand, corticofugal axons normally project toward the internal capsule and are supposed to directly interact with GABAergic interneurons originated into the ganglionic eminence via the cell adhesion molecule TAG-1.

It has been recently hypothesized that cortical and thalamic axons guide each others via interactions originating in the internal capsule. These interactions could involve surface molecules, secreted factors and/or extracellular matrix. In mice with mutations of transcription factors genes expressed in the cerebral cortex (Tbr1), dorsal thalamus (Gbx2), or both (Pax6), errors of corticothalamic axon pathfinding are invariably associated with errors of thalamocortical axon pathfinding. These errors occur in the internal capsule, outside of regions with altered gene expression. Conversely, both projections are intact in mice with mutations of transcription factors genes that disrupt development of the ganglionic eminence (Nkx-2.1, Dlx-1/2). These results suggest that cortical and thalamic axons interact in the internal capsule and there they exchange guidance cues necessary for reaching their targets.

Impairment of one or both these projection systems would result in defects of cortical differentiation as well as in misrouting of tangential migration of interneurons.

We used carbocyanine dye to study the development of corticofugal and thalamocortical pathways in mutant and in wild type embryos, as well as immunocytochemistry with antibodies against the neural adhesion molecules TAG-1 and L1 to mark cortical and thalamic axons respectively.

The results obtained indicate that in the absence of a functional Emx1 gene, those projection systems develop normally.

Thalamic axons coming from the thalamus and innervating the neocortex have also been implied in some later mechanisms of refinement and maintenance of the specificity of cortical areas.

Therefore, our results also confirm that even later stages of cortical arealization are not affected by Emx1 gene deletion.

4.1.6 Hypothetical redundancy and/or compensation between Emx genes: future work.

Our study on the phenotypic consequences of Emx1 gene inactivation during mouse embryogenesis clearly shows that Emx1 is not necessarily required for the proper development of the CNS and in particular of the cerebral cortex.

The reasons for the absence of obvious brain developmental abnormalities in $Emx1^{-/-}$ mice are still not known.

However, we hypothesize that the loss of Emx1 functions has been compensated for by other genes especially those having an overlapping expression pattern. In this regard, it is reasonable to assume that the other member of the Emx gene family, Emx2 could play such a role.

In order to establish whether there is a functional redundancy between Emx1 and Emx2 genes, we intend to repeat the experiments pertaining to the formation of the neocortex on $Emx1^{-/-}Emx2^{+/-}$ in parallel with $Emx1^{-/-}Emx2^{+/+}$ mice.

Our hypothesis is that the defects in the developing cortex of these animals will be more pronounced that those in the $Emx1^{-/-}$ mutants. In other words, without a copy of Emx2 gene, its compensation will be less efficient leading to the appearance of a more penetrant mutant phenotype.

4.2 THE STUDY OF THE ADULT Emx1 NULL CEREBRAL CORTEX REVEALS CYTOLOGICAL AND FUNCTIONAL ABNORMALITIES

4.2.1 Reduction of GABAergic interneurons in the adult $Emx1^{-/-}$ cortex

Emx1 gene expression in the dorsal telecephalon starts early during embryogenesis, and persists after birth in the mature cerebral cortex and hippocampus.

This suggests that the gene might have roles not only in the embryonic period of neocorticogenesis, but also during the postnatal refinement of the cerebral cortex and the maintenance of its functionality during the adult life.

Our analysis on the development of the neocortex in mice lacking a functional Emx1 gene has not shown significant abnormality.

Therefore, we hypothesized that the functions of this gene during embryogenesis, are redundant with those of other genes; so that, in the absence of Emx1, processes of neocoticogenesis and subsequent differentiation of the cortex can normally take place.

Emx1 gene is exclusively expressed by pyramidal neurons from the early stages of embryonic life to adulthood, and therefore can be reliably used as a marker of the pyramidal cell lineage. During development, cortical pyramidal neurons originate in the ventricular zone of the dorsal tencephalon, and utilize radial glia processes to migrate into the cortical plate.

The finding that Emx1 is specifically expressed by pyramidal neurons both in developing and mature cerebral cortex, suggests that Emx1 could be involved in the specification and maintenance of the pyramidal phenotype.

On the other hand, the vast majority of the cortical inerneurons arise from the ganglionic eminence of the ventral telencephalon (Anderson et al., 1997; Lavdas et al., 1999). These neurons appear to follow the corticofugal fibre system to reach their position in the developing neocortex.

We analyzed the density and the laminar distribution of the two neuronal cell types, the pyramidal and the nonpyramidal neurons in the neocortex of Emx1 mutant mice, using glutamate and GABA immunocytochemistry respectively.

In agreement with earlier reports, we found that glutamate-labelled neurons are present throughout the entire thickness of the cerebral cortex and absent in layer I, whereas GABA-containing cells are scattered in the cortex both in mutant and wild type animals.

Cell counts at three cortical areas along the rostrocaudal axis (motor, somatosensory and visual cortex) revealed no significant difference in the number of pyramidal neurons between the two groups of animals. However, counts of GABAergic interneurons showed statistically significant difference between mutant and wild type littermates, but only at the level of somatosensory and visual cortical areas.

Such change in the inhibitory interneuron population is very likely to have major effects in the establishment of neuronal circuits in the cortex and in a variety of functional and behavioral processes.

4.2.2 Tangential migration is impaired in Emx1$^{-/-}$ mice but with a very low penetrance

We detected a reduction of GABAergic interneurons in adult mutant cortices. Therefore we decided to investigate when this reduction first becomes apparent and if it is the consequence of a developmental defect.

To this purpose, we examined embryos between E14.5 and birth, when the process of tangential migration takes prominently place. At the same, we repeated the GABA immunocytochemistry at P0 and P20.

We used immunocytochemistry with specific molecular markers for tangentially migrating cells as well as for TAG-1, whose expression by corticofugal axon provides a guide for these incoming cells. We found that defects in the tangentially migrating cells can occur in the absence of a functional Emx1 gene but just in a small percentage of mutants, suggesting that the deletion of Emx1 gene does not affect the tangential migration of cortical interneurons. In most cases, therefore, other factors with redundant functions might compensate for the absence of Emx1.

On the other hand, the defects observed in the tangentially migrating calbindin-containing cells, represent a very low penetrant phenotype, that, however, might underlie some peculiar functions played by Emx1 in this process.

Moreover, at P0 no statistically significant difference is detected in the number of GABA-containing cells between Emx1 mutant and wild type littermates, meaning that the reduction of these cells becomes significant during the postnatal life.

4.2.3 Hypotheses and future work

Our findings suggest that the significant reduction of GABA-containing cortical cells occurs after birth.

One possibility is that Emx1, which is expressed by pyramidal neurons, is up-stream of a developmental cascade that influences also the development and maintenance of GABAergic cells even if these cells do not specifically express Emx1.

A second possibility is that tangential migration is impaired during embryogenesis, but the defects are subtle and not easy to be detected by immunocytochemistry: the specific markers could still be expressed by tangentially migrating cells, and that would be the reason why we could not observe differences in the vast majority of mutant embryos analysed. Nevertheless, other subtle mechanisms could be impaired.

Therefore, to study in deeper detail the process of tangential migration from the basal forebrain into the $Emx1^{-/-}$ neocortex, we intend to use DiI in cultured slices of embryonic forebrain of mutant and wild type mice, to reveal migration from the ganglionic eminence. Specifically, crystals of DiI will be placed in the ganglionic eminence, and the pattern of migration and disposition in the cortex of individual labelled-cells will be followed using time-lapse confocal microscopy.

In our opinion, these experiments would clearly indicate whether the observed reduction in the density of GABAergic neurons is because some cells fail to migrate to the cortex, and therefore there are subtle changes in the migratory path followed by these neurons that ultimately lead to their reduction during post-natal life, or whether it is due to a downregulation of the neurotransmitter by cortical neurons.

The third possibility is that GABAergic cells do not really disappear from the mutant cortex, and this would be in agreement with the fact that we could not detect any decrease in the overall neuronal density of the cortex in Nissl-stained sections. On the contrary, a decline in the expression of GABA would occur with age, supporting our observations at P0, when we could not see difference in the number of GABAergic cells between mutant and wild type animals.

To test this hypothesis, we have already started to dissect GABAergic cell population in smaller subpopulations distinguishable because of the expression of specific calcium binding proteins: calbindin, calretinin and parvalbumin.

In figure 4.1, it is shown an example of immunocytochemistry with antibodies against calbindin and calretinin in frontal sections of adult brains both mutant and wild type: the morphology and distribution of these cells was indistinguishable between the two groups of animals.

We also counted both calbindin and calretinin positive cells, but we could not detect any statistically significant difference comparing wild type and mutant animals.

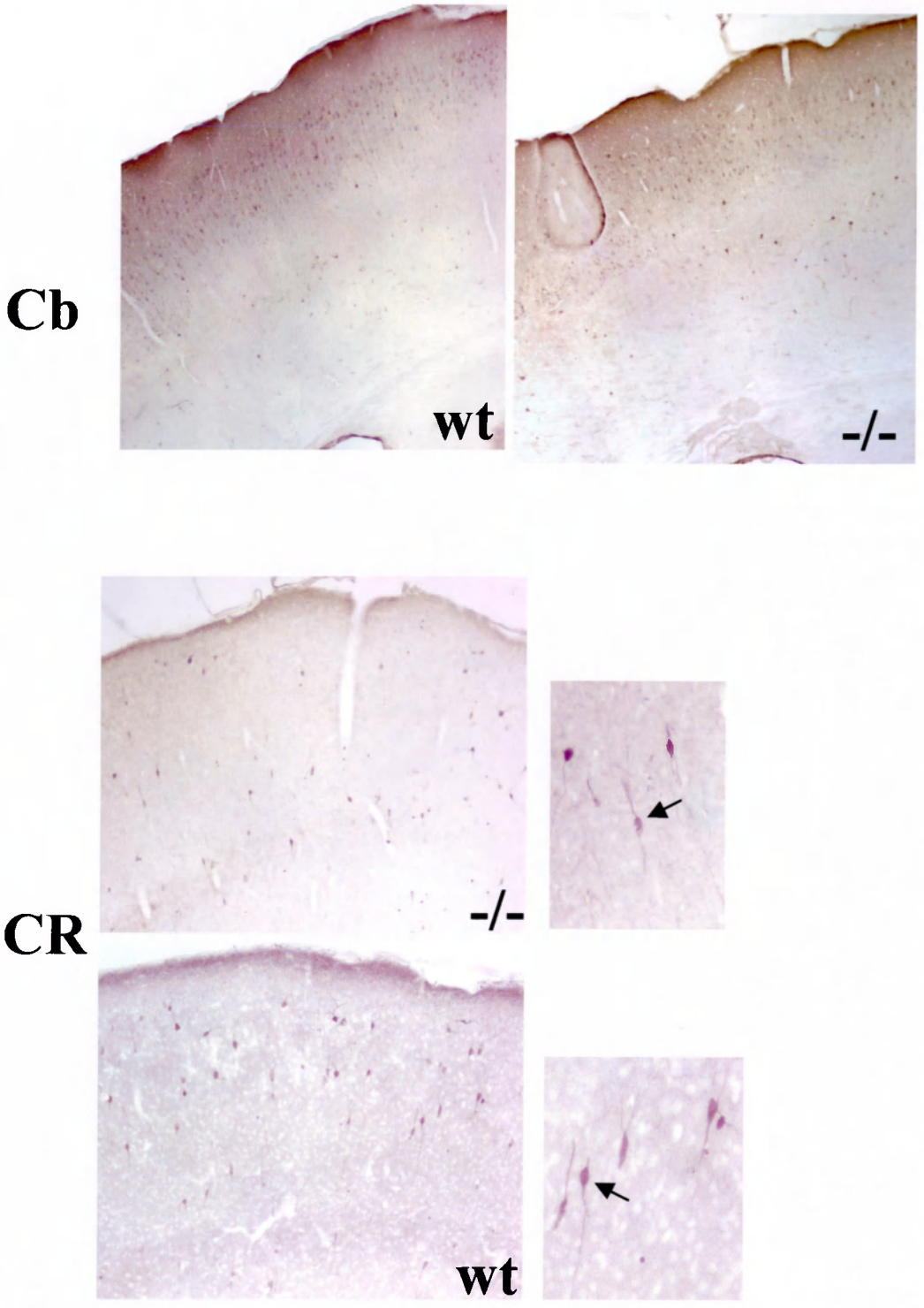

Figure 4.1

Calbindin and calretinin immunocytochemistry on frontal section of Emx1 mutant and wild type brains. Calbindin-positive cells are essentially located in the upper layers of the cortex (II, III and IV) both in mutant and in wild type brains. Calretinin-positive interneurons are scatterd throughout the cortex and exhibit a characteristic bipolar morphology (arrows). The difference in the number of calbindin positive cells, as well as calretinin-containing cortical interneurons, was not statistically significant between wild type and mutant brain (not shown).

These preliminary data suggest that, at least, those two subpopulations of GABAergic interneurons are not depleted in mutants, supporting the hypothesis that it is the neurotransmitter expression to be impaired.

Nevertheless, this analysis needs to be completed by studying other cellular subpopulation.

4.3 EMX1 NULL MUTANT MICE EXHIBIT AN EPILEPTIC-LIKE PHENOTYPE

As already reported, we confirmed that Emx1 mutant mice grow normally and do not have obvious anatomical or histochemical abnormalities in the brain.

The reason for the absence of obvious brain developmental abnormalities in Emx1 knockout mice is not known, and it is possible that the loss of the Emx1 function has been compensated for by redundant functions of other gene, especially those having an overlapping expression pattern.

Another possibility is that these mice have developmental defects or neurochemical changes that could not be detected by gross anatomical or histological methods. In this context, it has been reported that some homeobox genes are required for the development and differentiation of GABAergic neurons (Goridis and Brunet, 1999).

We have found that adult Emx1 null mutant mice exhibit a significant reduction of cortical GABAergic interneurons.

Whether or not Emx1 mutation causes a diminution of GABAergic cells or just a reduced synthesis of the neurotransmitter will be matter of the next analysis.

4.3.1 EEG recordings reveal altered electrical activity in the brain of Emx1 mutant mice

The reduction observed in the number of GABAergic interneurons prompted us to investigate if it was related to an altered electrical activity of the mutant brain.

Therefore, we obtained EEG recordings from adult $Emx1^{-/-}$ and wild type mice. Examination of these recordings showed epileptic-like activity characterized by abnormal spikes in all $Emx1^{-/-}$ animals. Moreover, we find that the immediate early genes *c-fos* is strongly activated in the cortex and hippocampus of mutants; by contrast almost no activation of *c-fos* is detectable in normal brains.

These results clearly demonstrate that in the deletion of Emx1 gene in mouse correlates with an epileptic activity of the brain.

4.3.2 Abnormal amino acid neurotransmitters release in the Emx1$^{-/-}$ cortices

We have found that a reduction of GABAergic neurons in Emx1 deficient cortices, that is likely to alter the formation and/or the functionality of neuronal circuits in those cortices.

We decided therefore to test if Emx1 gene influences the signalling pathways of GABA and possibly other neurotransmitter systems in the mutant cortex. On the other hand we also intended to verify if the reduction, that we observed in the GABAergic cell population, was due to a decline in the expression of the neurotransmitter and not only to the reduction of the cells.

To this purpose, we recently started to measure by HPLC the quantity of GABA and other amino acid neurotransmitters released by Emx1$^{-/-}$ cortical crude synaptosomes.

In line with the observed reduction of cortical GABAergic neurons, we found a significant decrease (approximately 25%) of both spontaneous and depolarization-evoked efflux of GABA in knockout mice when compared to wild type ones.

This result might explain the altered, epileptic-like electric activity recorded in the cortex of these animals, given the key role that the inhibitory amino acid plays in balancing neuronal excitation throughout the CNS.

Interestingly, we have also observed a marked decrease (approximately 40%) in the basal and evoked efflux of aspartate and glutamate, the two major excitatory amino acid neurotransmitters often reported to be co-localized and co-released by neuronal cells.

At present, this result is difficult to explain since it would seem that pyramidal neurons (the major glutamatergic neuronal population in the cerebral cortex) are not compromised in knockout mice, although we have not carried out a detailed quantitative and morphological analysis. However, these are the neurons where the Emx1 gene is selectively localized in the cerebral cortex of normal mice.

Therefore, we are tempted to hypothesize that the lack of Emx1 gene might not influence a correct migration and development of glutamatergic pyramidal neurons in the cortex but might affect their functional properties, thus leading to impairment of glutamate release. In any case, how a selective genetic defect (Emx1 deletion) in

glutamatergic neurons results in a marked loss of GABAergic cortical interneurons (which do not express the EMX1 gene) is difficult to understand and represents an intriguing issue.

However, several lines of evidence have demonstrated that glutamate, through interaction with its ionotropic and metabotropic receptors, is a crucial factor in triggering neuronal cell differentiation and maturation (Mayer and Westbrook, 1987; Pearce et al., 1987; Mattson et al., 1988; Rajan and Cline, 1998; Hannan et al., 2001; Mion et al., 2001). Thus, deficiency of glutamate availability during neurogenesis might have dramatic effects on neuronal development and/or maintenance.

This, in turn, correlates with the expression of Emx1 gene exclusively in pyramidal glutamatergic neurons.

4.4 EMX1 AND THE HUMAN EPILEPSY

Based on our data in mouse and on the evolutionary conservation of Emx genes among species and in particular Mammals, we hypothesized a role for the human EMX1 in the pathogenesis of some epileptic syndromes.

In the present study, we identified a mutation in the EMX1 homeobox gene in the affected members of a large family with an idiopathic generalized epileptic syndrome. All patients were heterozygous for the observed mutation.

Idiopathic generalized epilepsy is a genetic disorder with complex inheritance and in clinical practise it is fairly common to encounter a constellation of IGE syndromes within the same pedigree. The list of epilepsy recently proposed by the International League Against Epilepsy (ILAE) includes three so-called "syndromes of families" in addition to the classical "syndromes of individuals": generalized epilepsy with febrile seizures plus (GEFS+), familial focal epilepsy with variable foci, and idiophatic generalized epilepsy with variable phenotypes (Engel, 2001).

While the first two conditions are widely accepted (Scheffer and Berkovich, 1997; Scheffer et al., 1998; Scheffer and Berkovich, 2001), the third, which includes Juvenile Mioclonic Epilepsy (JME), Epilepsy with pure Generalized Tonic-Clonic Seizures (EGTCS) and juvenile absence epilepsy, represent a new addition currently under consideration. In this regard, the two IGE phenotype observed in the large family reported in this work might be viewed as part of this new family syndrome-idiopathic generalized epilepsy with variable phenotype.

It has also to be mentioned that epilepsies with Mendelian inheritance represent only a minority of the genetically determined epilepsies. Therefore, our findings represent a new insight into this issue.

Moreover, we observed that Emx1 null mice exhibit an epileptic-like phenotype presumably due to the reduction of cortical inhibitory interneurons.

These findings provide new insight into the mechanisms that underlie epileptogenesis. Indeed, idiophatic epilepsy may be viewed development disorder in which the selective inactivation of a homeogene leads to a reduction of inhibitory interneurons in the cerebral cortex and, hence, to increased neuronal excitability.

In order to elucidate the functional consequences of the EMX1 missense mutation identified in humans, it will be interesting to produce and analyse knock-in mice carrying the same mutation. This kind of study will give more direct information about the molecular mechanism by which Emx1 gene mutation affects the electrical properties of the brain.

Nevertheless, it is difficult to predict the functional consequences of this mutation, mostly because the molecular pathways triggered by EMX1 protein are still not known.

It has also to be mentioned that epilepsies with Mendelian inheritance represent only a minority of the genetically determined epilepsies. Therefore, our findings represent a new insight into this issue.

Finally, it is important to mention that, previously identified "epilepsy" genes account for only a small proportion of their associated syndromes. In fact, we have analyzed in 11 sporadic patients and one more two-generation family, with diagnosed idiophatic generalized epilepsy but we did not find the G→A mutation in the second exon of EMX1.

Therefore, the genetic basis of these conditions is likely to be highly heterogeneous given the rarity of mutations in genes so far implicated.

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